Molecular and Cytogenetical Dissection of the Rye (*Secale cereale* L.) Genome Structure

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ABSTRACT

Rye (*Secale cereale*. L) is an important crop mainly grown in Europe. It is also a valuable genetic resource in the improvement of wheat for its excellent biotic and abiotic stress tolerance. The insufficient number of PCR-based molecular markers and the lacking of dissection lines for rye have been hindering the development of its cytological or physical maps. The scarcity of rye physical maps have correspondingly restricted progress in genetic and genomic analyses of rye. The objectives of this study were to develop many PCR-based markers specific to each of the rye chromosomes, to produce rye dissection lines induced by the gametocidal system in the wheat background, to test the authenticity of a mass of rye SSR markers designed from the sequence information of flow-sorted rye chromosomes, and to map the rye specific SSR markers to individual chromosomes of rye with wheat-rye addition and substitution lines.

From previously-reported wheat PLUG markers, I chose 144 markers (primer sets) that are distributed in various chromosomal regions (bins). Through PCR analysis, I obtained 110 (76.4%) markers that showed rye-specific PCR amplification. Using a complete series of chromosome addition and substitution (for 3R) lines and 13 telosomic lines of common wheat including 2RS, 3RS, and 5RS that were developed in this study, I successfully mapped 79 rye-specific PLUG markers to the short or the long arm of the individual rye chromosomes. Considering the chromosomal bin locations of the 79 PLUG markers in wheat, I deduced a syntenic relationship between rye and wheat chromosomes, and suggested the occurrence of previously-unreported chromosomal rearrangements in the rye genome with reference to the cytologically visible chromosomal gaps of the rye chromosomes.

The gametocidal chromosomes have been known to cause chromosomal breakage in rye chromosome 1R added to common wheat. Stimulated by this fact, I introduced two different gametocidal chromosomes, 2C and $3C^{SAT}$, into the other six rye chromosome addition and substitution (for 3R) lines of common wheat. After

backcrossing, I successfully produced critical plants that were disomic for the rye chromosome and monosomic for gametocidal chromosome, so called chromosome-mutation-inducing plants or lines, for 2R to 6R except for 5R and 7R, and semi-critical plants, monotelodisomic additions of one whole and one telosome of the rye chromosome plus a gametocidal chromosome, for 5R and 7R. In the selfed progenies of these critical plants for 2R, 3R and 6R, I obtained the double disomic lines that are disomic for both rye chromosome and gametocidal chromosome. During the production of these lines, I found structural changes such as deletions and translocations involving every rye chromosome. Thus, it was demonstrated that dissection lines for every rye chromosome in common wheat can be produced by the gametocidal system.

The high level of polymorphism of SSRs was expected to facilitate marker development in rye. Based on the chromosome-specific survey sequences from sorted rye chromosomes, more than 26,000 SSR primer pairs were designed in the present study. By preliminary PCR analysis using 96 primer pairs, 49 from genic regions and 47 from non-genic regions, in the wheat-rye addition and substitution (for 3R) lines, I obtained 35 rye specific SSR markers, 22 (46.8%) from non-genic regions and 13 (26.5%)from genic regions. Hence I concluded that non-genic SSRs are more suitable for developing rye-chromosome specific markers than genic SSRs. and chose another 2,016 primer pairs from non-gene regions (288 per chromosome from the sorted rye chromosome sequences) for further PCR analysis. I successfully obtained 822 SSR markers that are specific to single rye chromosome (75 on 1R 125 to 2R, 124 to 3R, 135 to 4R, 130 to 5R, 105 to 6R, and 128 to 7R). Thus, all these PCR-based rye specific markers, together with those aneuploid lines of rye developed in this study, would become invaluable tools and materials for the genetic and physical mapping of the rye genome and also for other genomic studies of rye.

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LIST OF ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
BAC	Bacterial artificial chromosome
bp	base pair
CAPS	Cleaved amplified polymorphic sequences
cDNA	complementary deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
CTC	Circulating tumor cell
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
FC	Flow cytogenomics
Gc	Gametocidal chromosome
GISH	Genomic *in situ* hybridization
IMP	Inter-MITE polymorphism
IRAP	Inter-retrotransposon amplified polymorphism
ISH	*in situ* hybridization
Mbp	Megabase pair
nt	nucleotides
QTL	Quantitative trait loci
PCR	Polymerase chain reaction
PLUG	PCR-based landmark unique gene
RAMP	Randomly amplified microsatellite polymorphisms
RAPD	Random amplified polymorphic DNA
RAP-PCR	RNA fingerprinting by arbitrarily primed PCR

RBIP	Retrotransposon-based insertion polymorphism
REMAP	Retrotransposon-microsatellite amplified polymorphism
RFLP	Restriction fragment length polymorphism
RH	Radiation hybrid
RNA	Ribonucleic acid
SCAR	Sequence characterized amplified region
SNP	Single nucleotide polymorphism
SRAP	Sequence-related amplified polymorphism
S-SAP	Sequence-specific amplified polymorphism
SSCP	Single strand conformation polymorphism
SSLP	Simple sequence length polymorphism
SSR	Simple sequence repeat
SSRP	Simple sequence repeat polymorphism
STR	Short tandem repeats
STS	Sequence tagged site
TAE	Tris-acetate ethylenediaminetetraacetic acid
TD	Transposable display
TE	Tris-Ethylenediaminetetraacetic acid
TRAP	Target region amplification polymorphism
YAC	Yeast artificial chromosome

GENERAL INTRODUCTION

Rye

Rye (*Secale cereale* L.) is one of the most recently domesticated cereals belonging to the tribe Triticeae in grass (Poaceae) family, with a likely origination in East Turkey (Sencer and hawkes 1980) and in South West Asia (Bushuk 2001). It is closely related to barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) (Kreis et al. 1985; Castillo et al. 1994) and is an important diploid species (2n = 14) grown extensively as a grain and as a forage crop. Its production sites are mainly distributed in most of Europe, and in parts of Northern America, South America and Asia (from Wikipedia, the free encyclopedia, Rye). According to FAO's statistical data, the production of rye was about 13.3 million metric ton in 2005 with a decreasing trend year by year in most of the production nations. However, it is still a staple crop together with wheat and barley and mainly used for making bread which is continuing and growing populous for its distinctive flavor, taste and eating quality (Lorenz 2003). Rye is also a significant forage food to livestock and other animals (Hodgson 1976; Franzluebbers 2007; Yolcu et al. 2009), and also used for brewing alcoholic drink, like rye whiskey and rye beer. Undoubtedly, rye is closely interwoven with human history as a staple crop together with wheat and barley, and it possibly belongs to the Neolithic founder crops that built western agriculture (Kilian et al. 2009). In rye, taxonomy and phylogenetic relationships are controversial and have been discussed for over seven decades possibly due to its outcrossing nature and the interspecific-fertility (Vavilov 1917; Stutz 1972; Sencer and Hawkes 1980; Hammer et al. 1987; Frederiksen and Petersen 1997, 1998; Jaaska 1998; Zohary and Hopf 2000; Chikmawati et al. 2005). At present, the cultivated rye is classified into the first group out of the three groups of taxa in *Secale*—the annual outcrossing *S. cereale* complex.

Besides as an important staple crop, rye is an invaluable genetic germplasm due to its excellent tolerance to biotic and abiotic stresses (Mohammadi et al. 2003; Bartoš et al. 2008). Rye also has an excellent tolerance to low temperatures and the ability to

achieve relatively high yield under environmental conditions in which other crops perform poorly (Madej 1996). As a genetic resource, rye plays a significant role for wheat improvement, and it is also a donor of the R genome to a man-made species triticale (X *Triticosecale* Wittmack) (Milczarski et al. 2011). In addition, rye is widely used by breeders mainly because of its favorable features such as nutrient efficiency and resistance to diseases (Bolibok-Brągoszewska et al. 2009), e.g. resistance genes to leaf rust (*Lr26*), stem rust (*Sr31*), stripe rust (*yr9*) and powdery mildew (*pm8*) (McIntosh 1988; Baum and Appels 1991).

Rye genome constitution

Rye (2n=2x=14, genome formula RR), a diploid outbreeding species, diverged from a common ancestor with wheat (2n=6x=42, genome formula AABBDD) only six million years ago (Huang et a. 2002). However, its karyotypes exhibit dramatic differences with those of wheat in size and structure (Gill and Friebe 2009).

A karyotype is the number and appearance of chromosomes in the nucleus of a eukaryotic cell. The term is also used for the complete set of chromosomes in a species, or an individual organism (Stebbins 1950; White 1973). For chromosome karyotyping, geneticists paid attention to chromosome length, the position of the centromeres, banding pattern, any differences between the sex chromosomes, and any other physical characteristics (King et al. 2006). Furthermore, the preparation and study of karyotypes is one of the main parts of cytogenetics. The rye chromosomes can be distinguished easily from wheat chromosomes in wheat × rye hybrids because of two apparent cytological characteristics. One of the characteristics is that the rye chromosomes are longer in size because the rye genome (C-value, about 9.5 pg) (Bennett and Smith 1976) is about 33% larger in size than the diploid wheat genome (C-value, around 6 pg) (Arumuganathan and Earle 1991; Gill and Friebe 2009). The other is the presence of massive blocks of terminal heterochromatin in all rye chromosomes, which were demonstrated or depicted by some researchers (Lima-de-Faria 1952; Gill and Kimber 1974; Thomas and Kaltsikes 1974; Darvey and

Gustafson 1975; Mukai et al. 1992). At present, the parameters including the relative size of the terminal C-bands, presence/absence and pattern of minor interstitial bands, and arm ratios are key indexes for the cytogenetic diagnosis, for the identification of the individual rye chromosomes, telosomes and deletions. The set of 'Imperial' rye chromosomes added to common wheat Chinese Spring was considered as the standard rye chromosome set in the workshop report on rye chromosome nomenclature and homoeology relationships (Sybenga 1983). Using C-banding and *in situ* hybridization techniques together with diagnostic parameters, Mukai et al. (1992) presented karyotypic and molecular cytogenetic information, providing the detailed and complete characteristics of the rye chromosomes.

Comparative genomics is the study of the relationship between genome structure and function across different biological species or strains (Paterson et al. 2000). It is one of the major approaches used in the functional annotation of genomes. Comparative genomics is actually an important attempt to take advantage of the information provided by the signatures of selection to understand the function and evolutionary processes that act on genomes (Miller et al. 2004). While it is still a young field, comparative genomics has been a powerful and burgeoning discipline that will become more and more informative as genomic sequence data accumulate (Hardison 2003; Miller et al. 2004). Meiotic metaphase I pairing analysis (Naranjo et al. 1987; Naranjo and Fernández-Rueda 1991), RFLP analysis (Liu et al. 1992; Devos et al. 1993a, 1993b) and comparative analysis of genome relationships (Martis et al. 2012) revealed that the rye genome underwent a series of rearrangements after its divergence from the wheat and barley lineages with the exception of 1R. Evidently, most of the rye chromosome arms have been involved in one or more rounds of translocations or inversions.

Rye aneuploids

Rye aneuploid stocks in a broad sense include primary trisomics and telotrisomics (Zeller et al. 1977; Schlegel et al. 1986), and monosomic additions, disomic additions

and telosomic additions of rye chromosomes to wheat (Driscoll and Sears 1971; Mukai et al. 1992; Li et al. 2013). Also, deletions of rye chromosomes in the wheat background (Friebe et al. 2000; Tsuchida et al. 2008; Gyawali et al. 2009, 2010), and even monosomic additions of wheat chromosome to rye (Schlegel 1982) are included. Generally, primary trisomics and telotrisomics, and wheat monosomic addition to rye do not breed true and are highly sterile, which will make it difficult to study them (Gill and Friebe 2009). Instead, a series of additions, substitutions or translocations of rye chromosomes to wheat (Driscoll and Sears 1971; Mukai et al. 1992) have been important genetic materials for rye genome mapping. A complete series of 'Imperial' rye chromosome addition lines to hexaploid wheat cv. 'Chinese Spring' were developed by Driscoll and Sears (1971). For telosomic addition lines, to my knowledge, it is difficult to trace their initial source completely. Perhaps, the earliest comprehensive description about rye telosomic lines was reported by Mukai et al. (1992): they used 11 ditelosomic addition lines (1RS, 1RL, 2RL, 3RS, 4RS, 4RL, 5RS, 5RL, 6RL, 7RS, and 7RL), which were provided by T. E. Miller, IPSR, Cambridge Laboratory, U.K. However, the lines 2RS, 3RL, 5RS and 6RS were unavailable when the present research work was started. Fortunately, through the screening of the self-pollinated progeny of the monosomic addition lines of Imperial rye, Li et al. (2013) obtained three of the four unavailable telosomic addition lines, namely 2RS, 5RS and 6RS. Therefore, 13 of all 14 rye telosomic addition lines (except for 3RL) are available now. These rye telosomic lines are undoubtedly an exciting acquisition and will contribute greatly to the promotion of rye genetic and genomic analyses.

Thanks to the Gametocidal (Gc) system (Endo and Tsunewaki 1975) (see the next section below for details), the production of wheat-rye recombinant chromosome stocks have been accelerated, which has provided further opportunities for cytological or physical chromosome mapping (Rogowsky et al. 1993; Lukaszewski 2000, 2004). To date, many deletions have been developed by the Gc system in wheat (Endo and Gill 1996) and in wheat-barley addition lines (Shi and Endo 1999, 2000; Serizawa et al. 2001; Ashida et al. 2007; Sakai et al. 2009; Endo 2009; Sakata et al. 2010; Joshi et

al. 2011). For rye, Friebe et al. (2000) and Masoudi-Nejad et al. (2002) have used the Gc system in wheat-rye addition lines to induce chromosome rearrangements, including translocations and simple deletions; however, they have been little exploited. Hereafter, Tsuchida et al. (2008) and Gyawali et al. (2009, 2010) developed dissection lines including deletion and translocation lines of 1R on a large-scale, and constructed physical maps using the dissection lines and PCR-based molecular markers.

Gametocidal system

The gametocidal (Gc) system, which was first found by Endo (1975) during the production of alien cytoplasm substitution lines or alien chromosome addition lines in common wheat, has become an important tool for chromosome manipulation in wheat (Endo 2007). The Gc chromosomes are derived from different wild species of *Aegilops*, a genus related to wheat (*Triticum*). In the Gc system, the Gc chromosome is introduced into common wheat and into some wheat-Triticeae species addition lines, such as wheat-barley, wheat-rye additions, to induce random chromosome breakage leading to the generation of deletions and translocations involving the added alien chromosomes (Endo 2011). Figure **I** shows how the Gc system works as a genetic tool in chromosome manipulation: when introduced into rye-wheat addition lines, it causes chromosome breakage only in the gametes without the Gc chromosome. Till now, using the Gc system, many deletion stocks have been established for common wheat chromosomes (Endo and Gill 1996) and also for some alien chromosomes added to common wheat, i.e. most of the barley chromosomes (Shi and Endo 1999, 2000; Serizawa et al. 2001; Ashida et al. 2007; Sakai et al. 2009; Sakata et al. 2010; Joshi et al. 2011) and part of the rye chromosomes (Friebe et al. 2000; Tsuchida et al. 2008; Gyawali et al. 2009, 2010). Moreover, these deletion stocks have been used in the construction of cytological chromosome maps of DNA markers, such as RFLPs (Werner et al. 1992) and ESTs (Qi et al. 2004). Therefore, cytological mapping by the Gc system, so called Gametocidal mapping, will be undoubtedly an important way for

high-throughput gene mapping in the post-genomics era (Masoudi-Nejad and Endo 2008).

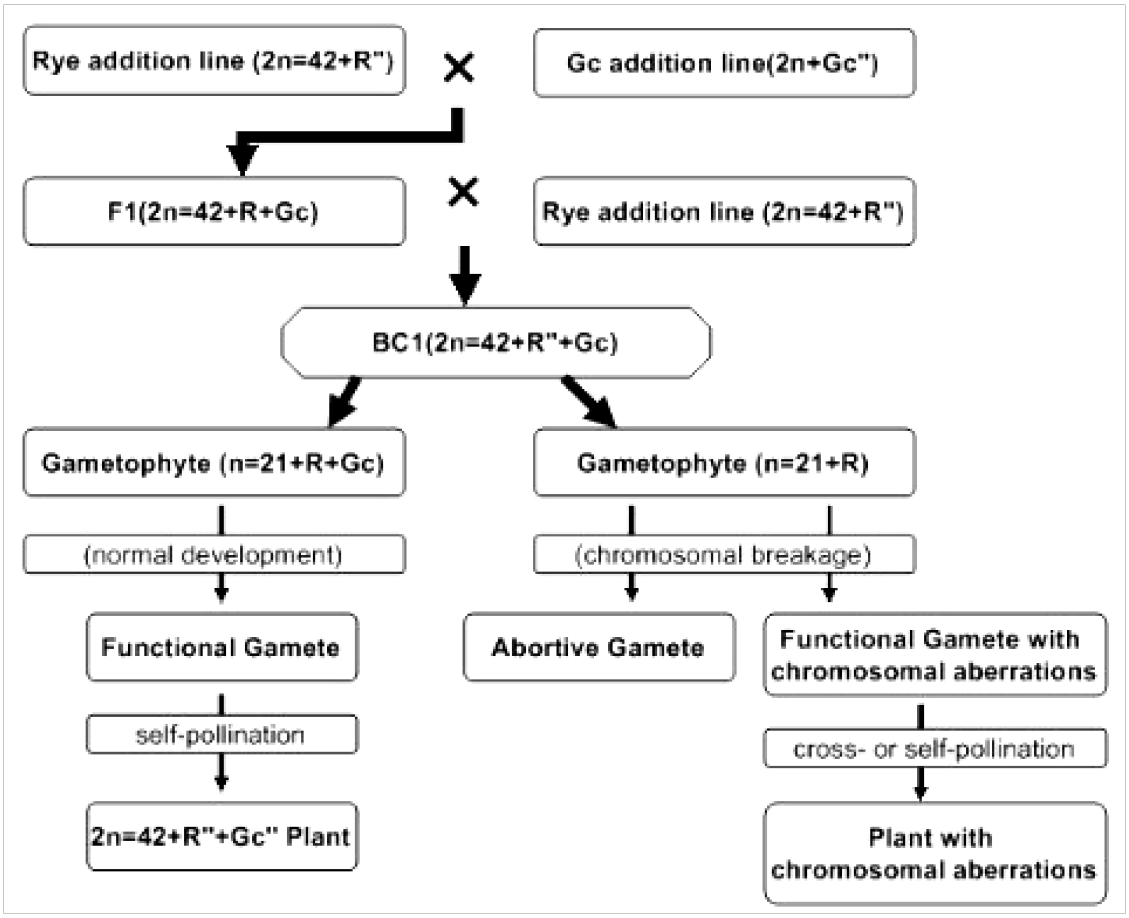


Fig. I Schematic illustration of the mechanism of the Gc system.

In situ hybridization

In situ hybridization (ISH) is a technique that uses a labeled complementary DNA or RNA strand, i.e. probes, to localize or identify specific DNA or RNA sequences in a portion or section of tissue (*in situ*), or, if the tissue is small enough (e.g. plant seeds, *Drosophila* embryos), in the entire tissue (whole mount ISH), in cells and in circulating tumor cells (CTCs) (from Wikipedia, the free encyclopedia, circulating tumor cell; McNicol and Farquharson 1997). It represents a unique technique combining molecular biological and histochemical techniques to detect gene expression in tissue sections and cytological preparations (Jin and LIoyd 1997). The

ISH technique was originally developed by Gall and Pardue (1969) and (independently) by John et al. (1969) to detect RNA-DNA hybrid molecules on cytological preparations of toad (the genus *Xenopus*) oocytes. At beginning, radioisotopes were the only labels available for nucleic acids, and autoradiography was the only way of detecting hybridized sequences. Moreover, since the technique of molecular cloning was unavailable in those days, *in situ* hybridization was restricted to those sequences that could be purified and isolated by conventional biochemical methods (e.g., mouse satellite DNA, viral DNA, ribosomal RNAs) (Eisel et al. 2002). Later, the emergence of molecular cloning and the improvement of radiolabeling techniques caused dramatic changes in ISH. The most typical examples were the successful detections of DNA sequences of a few hundred base pairs in metaphase chromosomes (Harper et al. 1981; Jhanwar et al. 1984; Rabin et al. 1984; Schroeder et al. 1984) and of mRNA molecules in low copy numbers in individual cells (Harper et al. 1986) by autoradiography. However, in spite of the high sensitivity and broad application of the *in situ* hybridization techniques at that time, their use was restricted to limited research laboratories because of the problems related to radioactive probes, such as meeting the requirements for safety measures, their limited shelf life and prolonged time for autoradiography. In reality, only after ISH underwent many modification and refinements in 1990s, it became a practical tool in basic scientific researches and clinical diagnosis. Since then, many variations of ISH procedures have sprung up (Wilcox 1993; Wilkinson 1993; Egger et al. 1994; Panoskaltsis-Mortari and Bucy 1995).

At present, hybridization probes (labeled nucleic acid molecules) can be prepared with stable nonradioactive labeling, which removes the key obstacles that hinder the general application of *in situ* hybridization. Furthermore, it is possible for combining different probes in one experiment (so called multicolor *in situ* hybridization). Hence, ISH is undoubtedly an effective technique for the diagnosis of infectious diseases (Myerson et al. 1984; Naber 1994; McNicol and Farquharson 1997), for the analysis of cytogenetics (Trask 1991; Schwarzacher and Heslop-Harrison 2000) and for the detection of gene expression (Singer and Ward

1982; Lawrence and Singer 1985; Wilkinson 1998; Lein et al. 2007). Generally, nonradioactive hybridization methods can be classified as two categories: direct hybridization and indirect hybridization (Eisel et al. 2002). In the direct method, the detectable molecule (reporter) is bounded directly to the nucleic acid probe so that probe-target hybrids can be visualized under a microscope. When antibodies against the reporter molecules are used, direct methods are converted to indirect immunochemical amplification methods. The following describes two commonly used and important techniques of indirect *in situ* hybridization.

Fluorescence *in situ* hybridization (FISH) is a molecular cytogenetic technique utilizing fluorescently labeled DNA probes to detect or confirm gene and chromosome abnormalities. It is a powerful and versatile research tool in genetic and genomic analysis and also an important adjunct to diagnosis and prognosis in clinical cytopathology (Tkachuk et al. 1991; Wolman 1997; Levsky and Singer 2003; Oliveira and French 2011). Like some other molecular techniques, FISH is based on the fact that complementary single-stranded nucleic acid molecules (DNA or RNA) could achieve hybridization each other (Sambrook et al. 1989) under suitable conditions. The most important characteristic of FISH is its ability to localize specific DNA sequences in intact nuclei and chromosome spreads. In plants, since its inception in the early 1970s (Miller 1995), FISH has become a powerful tool in cytological research. Thanks to the developments and improvements of high resolution FISH, the analysis of relatively long DNA molecules has become relatively routine by combining some innovative methods of molecular mapping and data sets processing (Hans de Jong et al. 1999). Furthermore, together with genomic *in situ* hybridization (GISH), FISH is widely used to determine genome structure in crop plants with complex and large genomes, such as wheat (Schwarzacher et al. 1992). Besides, barley FISH probe HvT01 (Belostotsky and Ananiev 1990), a subtelomeric repetitive sequence, was efficiently used to identify dissected barley chromosomes in the wheat background (Sakai et al. 2009). For rye, two repeat sequences of subtelomeric region, pSc119 and pSc200 (Bedbrook et al. 1980; Vershinin et al. 1995), were often used as FISH probes for the analysis of rye genome structure (Cuadrado et al. 1995; Wilkes et

al. 1995; Tsuchida et al. 2008; Gyawali et al. 2009, 2010; González-García et al. 2011).

Genomic *in situ* hybridization (GISH) is another valuable and effective ISH tool for studying genomic composition and interactions in interspecific and intergeneric hybrids (Schwarzacher et al. 1989; Jacobsen et al. 1995; Lim et al. 2003; Bi and Bogart 2006; Liu et al. 2009). This technique uses labeled whole genomic DNA as probes in *in situ* hybridization to detect genomes or chromosomes of related species. Since Schwarzacher et al. (1989) initially applied GISH to detect and distinguish chromosomes originating from the two parental genomes in a wide hybrid, this technique has been widely and successfully applied in many crops, such as rice (Apisitwanich 1999; Tan et al. 2005; Jin et al. 2006), wheat (Schwarzacher et al. 1992; Liu et al. 2007; Qi et al. 2008), barley (Pickering et al. 1997; Schubert et al. 1998; Shi and Endo 2000) and rye (Heslop-Harison et al. 1990; Masoudi-Nejad et al. 2002). GISH was used to identify alien chromosomes or segments in the process of hybridization, translocation breakpoints, chromosome pairing activity and the genome composition of polyploid plants. Today, GISH is a fast, sensitive, accurate and informative method in genetic and genomic analyses.

Chromosome mapping

Chromosome mapping is the process of determining the position of unique, identifiable morphological, biochemical and molecular landmarks, including some specific genes on specific chromosomes, and constructing a series of diagrams of each chromosome showing the relative positions of the genes (Verrity and Abbington 2008; from American Heritage Stedman's Medical Dictionary: chromosome mapping). Chromosome maps are very important and essential for genetics and genomics studies of any organisms, Hence chromosome mapping was one of the key research areas of the Human Genome Project completed in 2003. In general, there are two types of chromosome mapping, genetic (linkage) mapping and physical (cytological) mapping.

Genetic maps are the fundamental tools to identify features of phenotypes that linked to specific genetic loci and eventually DNA sequences or genes. It has mainly been used to identify useful quantitative trait loci (QTL). In addition, genetic maps are also essential for marker-assisted breeding selection, comparative mapping, high-resolution mapping and map-based cloning. So far, over 40 linkage maps with hundreds of molecular markers have been established in Triticeae (Lehmensiek et al. 2009). For rye, since Melz et al. (1992) developed a systematic linkage map, a series of genetic linkage maps with various markers have been constructed (for details, see Milczarsski et al. (2011) and Tyrka et al. (2011)).

Although the genetic linkage map, by measuring genetic distances between marker alleles or phenotypes in the region of interest, can address questions related to the structural organization of a genome, it provides a relatively reasonable indirect measurement for the underlying physical distance given in base pairs because the genetic distance only reflect the probability for the occurrence of a crossing-over during meiosis between two landmarks on a chromosome (Stein 2009). Furthermore, since recombination is not dispersed evenly along whole chromosomes, especially in Triticeae crop species, such as wheat, barley and rye, it would be inaccurate to convert recombination frequencies into bp-distances. Even more, some hot spots of recombination were found in gene-rich regions while the areas with low gene density or high constitution of heterochromatin may not show any recombination at all (Stein 2009). In this condition, it is urgent and essential to set up a novel mapping strategy for complementing those drawbacks.

Physical maps show the arrangement of the material, in the form of banding patterns produced by staining or the sequence of bases in the DNA, making up a chromosome or segment of a genome (from Encyclopedia, physical map). Normally, there are two main types of physical maps, differing vastly in scale and detail. One is the ordered-marker based physical map (Stein 2009), namely cytological map in cytogenetics and cytogenomics, which is a schematic representation of chromosomes indicating the arrangement of various specific markers including genes along the chromosomes in relation to chromosome banding patterns, characteristic structures

such as centromeres, telomeres, and (if any) secondary constrictions. Cytological maps are constructed mainly on the basis of the analysis of chromosomal aberrations, e.g. translocations and deletions. The other one is ordered-clone based physical map (Stein 2009), namely contig map, which is established by high-throughput generic approaches and shows the order of cloned DNA segments taken from a DNA library and fitted together to form a series of overlapping or contiguous segments, called a contig. Therefore, physical mapping can deliver absolute distances between genomic landmarks, providing direct access to any point in a genome and to the isolation of any interest gene (Stein 2009). Now, physical maps are useful tools for genome sequence assembly. However, compared to small genome model species, like *Arabidopsis thaliana* (Meinke et al. 1998), where whole-genome "shotgun sequencing" is sufficient, it is almost impossible to assemble billions of sequenced pieces of a large and complex total genome in order by "shotgun sequencing", even though using next-generation sequencing techniques. Thus, in the post-genomic era, physical mapping is a mandatory method to develop whole-genome reference sequences for large and complex genomes, such as those of the Triticeae crop species wheat, barley and rye (Masoudi-Nejad and Endo 2008; Stein 2009).

Generally in plants, physical mapping, based on Bacterial Artificial Chromosome libraries (BAC's, Shizuya et al. 1992), Yeast Artificial Chromosome libraries (YAC's, Burke et al. 1987), P1 phage contig and cosmid, has largely focused on some model species, such as Arabidopsis (*Arabidopsis thaliana* L.) and rice (*Oryza sativa* L.) with relatively small genomes, 150Mbp/1C (Bennett and Smith 1976; Goodman et al. 1995) and 430 Mbp/1C (Kurata et al. 1997), respectively. While in species with very large and complex genomes like bread wheat (16,937 Mbp/1C, Bennett and Smith 1976), barley (5100 Mbp/1C, Doležel et al. 1998) and rye (7917 Mbp/1C, Vicient et al. 1999), whose genome sizes are 35-fold, over 10-fold and ca. 18-fold larger than that of rice, respectively, the traditional physical mapping is limited because of the obstacles and constraints of the techniques themselves. Besides, although radiation hybrid (RH) mapping has made great progress on the studies of human and mammalian genome organization, it has a very limited

successful application in plants. Thanks to a unique genetic system—the Gc system as above described, developed by Endo (1975, 1988, 1990) could conquer most of these problems and most probably to serve as an alternative to RH mapping.

Over past 20 years, most of the cytological chromosome maps have been established in wheat and barley by using the Gc system, however, there are still very few cytological maps in rye except for 1R (Tsuchida et al. 2008; Gyawali et al. 2009, 2010). To my knowledge, there are two major reasons that caused the scarcity of rye physical maps. One is the lack of molecular markers, especially PCR-based markers. Rye is underrepresented in the sequence databases, with only 10,369 ESTs, in contrast to wheat 1,575,304 and barley 556,070 ESTs deposited in GenBank. Conversely, the lack of sequence information has limited marker development and gene cloning in this species (Bartoš et al. 2008). The other main reason is the scarcity of dissection lines comprising deletions and translocations, which were widely used to construct cytological maps in wheat and barley. Compared with wheat and barley dissection stocks, a substantial number of this dissection lines are available only for 1R in rye. Although a few deletion lines have been developed by Friebe et al. (2000) for other rye chromosomes except for 7R, their numbers were far from sufficient for chromosome mapping and genomic studies.

Molecular markers

Molecular markers, identified as genetic markers in genetics and sometimes called DNA markers, are the fragments of DNA or the signs along the DNA trail that pinpoint the location of desirable genetic traits or indicate specific genetic differences within the genome (from Wikipedia, the free encyclopedia. Molecular marker; Using molecular markers in plant genetics research). Over the past few decades, molecular markers revolutionized the entire scenario of biological sciences. Nowadays, DNA-based molecular markers have acted as versatile tools and have found their own position in various fields like taxonomy, physiology, embryology, genetic engineering, etc (Joshi et al. 1999).

In plants, molecular markers have now become an important tool in various aspects of plant genome analysis involved in taxonomy, phylogeny, ecology, genome mapping and plant breeding. Mapping and sequencing of plant genomes would help to elucidate gene function, gene regulation and gene expression (Mohan et al. 1997). The critical first step to mapping the genome is to find genetic markers. These markers should have unique sequences that provide orientation points in the genome landscape. Since Botstein et al. (1980) reported the first molecular marker technique, restriction fragment length polymorphism (RFLP) in the detection of DNA polymorphism, for the construction of genetic maps, many types of marker techniques have been developed. Generally, all basic marker techniques can be classified into two classes: (1) non-PCR-based techniques or hybridization-based techniques and (2) PCR-based techniques (Gupta et al. 1999; Agarwal et al. 2008).

Non-PCR-based techniques

The markers of non-PCR based techniques are hybridization-based molecular markers and mainly refer to RFLPs. Among all kinds of molecular markers developed, RFLPs were the first to be used in human genome mapping (Botstein et al. 1980) and later were adopted for plant genome mapping (Helentjaris et al. 1986; Weber and Helentjaris 1989) including that of rye (*Secale cereale*) (Korzun et al. 1996, 1997, 1998 and 2001; Börner and Korzun 1998; Wang et al. 1991; Devos et al. 1993a, 1993b; Philipp et al. 1994; Rognli et al. 1992). In RFLP, DNA polymorphism is detected by hybridizing a chemically labeled DNA probe to a Southern blot of DNA digested by restriction endonucleases, resulting in differential DNA fragment profiles.

The RFLP markers are relatively high in polymorphic, co-dominantly inherited and reproducible, and they are considered superior because of their presence throughout the plant genomes, their high heritability and locus specificity. RFLP, however, is not widely used nowadays because it is time-consuming and labour-intensive, and requires expensive and radioactive/toxic reagents, and also high quality genomic DNA in quantity.

PCR-based techniques

Since the moment of the invention of polymerase chain reaction (PCR) (Mullis and Faloona 1987), many approaches for the development of molecular markers based on PCR have been devised, mainly thanks to its apparent simplicity and high success rates. The molecular markers based on PCR offer the potential to reduce the time, effort and expense required for molecular mapping. PCR-based techniques can further be subdivided into two subgroups (Agarwal et al. 2008): arbitrarily primed PCR-based techniques (or sequence non-specific techniques) and sequence targeted PCR-based techniques.

Arbitrarily primed PCR-based techniques primarily include random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP). Sequence specific PCR-based markers mainly comprise microsatellites and single nucleotide polymorphism (SNPs) (Agarwal et al. 2008). Additionally, with the advancement of marker techniques and discoveries plus in-depth studies of genome, molecular marker techniques have been greatly enhanced over the past two decades. These advanced techniques are not only an amalgamation of the advantageous characteristics of several basic techniques but also an incorporation of modifications in the methodology to increase the sensitivity and in the resolution to detect genetic discontinuity and distinctiveness. To date, many kinds of molecular marker techniques have been developed. They consist of organelle microsatellite, namely chloroplast microsatellites (Powell et al. 1995; Provan et al. 2001) and mitochondrial microsatellites (Rajendrakumar et al. 2007), sequence characterized amplified regions (SCAR) (Paran and Michelmore 1993; McDermott et al. 1994), sequence tagged sites (STS) (Olson et al. 1989), expressed sequence tags (EST) (Adams et al. 1991), cleaved amplified polymorphic sequences (CAPS) (Komori and Nitta 2005), randomly amplified microsatellite polymorphisms (RAMP) (Wu et al. 1994), sequence-related amplified polymorphism (SRAP) (Li and Quiros 2001), target region amplification polymorphism (TRAP) (Hu and Vick 2003), single strand conformation polymorphism (SSCP) (Orita et al. 1989), transposable elements-based

molecular markers (Finnegan 1989; Grzebelus 2006), retrotransposon-based molecular markers (including (a) inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP) (Kalendar et al. 1999), (b) sequence-specific amplified polymorphism (S-SAP) (Waugh et al. 1997), (c) retrotransposon-based insertion polymorphism (RBIP) (Flavell et al. 1998)), transposable display (TD) (Van den Broeck et al. 1998), inter-MITE polymorphism (IMP) (Chang et al. 2001), RNA-based molecular markers (including (a) cDNA-SSCP (Cronn and Adams 2003), (b) RNA fingerprinting by arbitrarily primed PCR (RAP-PCR) (Welsh et al. 1992), (c) cDNA-AFLP (Bachem et al. 1996)). Each of these marker techniques has its advantages and special applications in plant genetic and genomic analysis. In conjunction with the present study, I would like to make two detailed descriptions of two basic molecular marker technique, namely simple sequence repeats (SSRs) and PCR-based Landmark Unique Gene (PLUG) as follows.

SSRs, also referred to as microsatellites or short tandem repeats (STRs), are ubiquitous in eukaryotic genomes and initially used in mammalian genome studies (Tautz and Renz 1984; Weber and May 1989). SSR polymorphism (SSRP) reflects the polymorphism based on the number of repeat units in a defined region of the genome being investigated (Jacob et al. 1991; Litt and Luty 1989; Weber and May 1989). The high level of polymorphism, relative to RFLPs and RAPD, combined with a high interspersion rate makes SSR an abundant source of genetic molecular markers (Gupta et al. 1999). In addition, microsatellites are highly popular genetic markers because of their codominant inheritance, enormous extent of allelic diversity, and the ease of assessing SSR size variation by PCR with pairs of flanking primers. However, only after Condit and Hubbel (1991) firstly reported microsatellites in tropical trees, scientists found that they are abundant in higher plants as well (for details see Gupta et al. 1996). Later, Akkaya et al. (1992) developed length polymorphisms of SSR in soybean, which established a new source of PCR-based molecular markers for other plant genomes. To date, microsatellites have been highly studied in wheat and barley. For wheat, after two genome-specific SSR markers were developed by converting two

microsatellite sequences into PCR-based markers (Devos et al. 1995), the potential and practicality of SSR as genetic markers in wheat were investigated and carried out in genome mapping and marker-assisted breeding (for details see Gupta et al. 1999). In barley, many high density maps have been constructed with SSR markers in recent years (Ramsay et al. 2000; Kota et al. 2001; Holton et al. 2002; Karakousis et al. 2003; Varshney et al. 2007; Hearnden et al. 2007). For rye, although a certain number of SSR markers were also developed for map construction (Saal and Wricke 1999; Khlestkina et al. 2004; Milczarski et al. 2007), the scant number is still not meet the demand for constructing high-density rye genetic or cytological maps, which are further hindering rye genomic analyses. Therefore, to develop a mass of SSR markers would be imperative for rye genome studies.

On the other hand, a fact that homoeologous loci are not always available (Bryan et al. 1997; Röder et al. 1998; Stephenson et al. 1998) restricts, to some degree, the application of microsatellites to intraspecific and intragenomic analyses, and the loss of homoeologous loci almost completely limited the use of SSR marker for comparative analyses or for introgression studies involving wild species related to wheat (Gupta et al. 1999). As a technique to overcome such drawbacks, I introduced another marker technique, namely PLUG, in the present study.

The PLUG marker system was firstly developed by Ishikawa et al. (2007) in wheat by using the similarities in gene structure between rice and wheat. It is well known that EST-PCR markers normally represent specific products from target genes, and are therefore effective tools for genetic and genomic analysis. However, in allohexaploid wheat, EST-PCR markers often yield multiple products derived from paralogous genes, as well as from homoeologous genes, which restrict the mapping of EST-PCR molecular markers on wheat chromosomes. To overcome such problems, it may be practicable if such amplified PCR products include intron sequences, which are more polymorphic in terms of insertions/deletions and base substitutions than exon sequences are (Bryan et al. 1999). However, genomic sequence data for wheat are so limited that it is difficult to predict the accurate location of introns. Therefore, based on the sequence conservation of orthologous genes, Ishikawa et al. (2007)

developed the PLUG system to design primers within separate exons of an EST, which can be inferred from the genomic sequences of rice in the RAP database for which intron-exon structures are indicated (Rice Annotation Project 2008). Since insertion/deletion polymorphisms are supposed to be high in intron sequences from wheat homoeologous genes, the PLUG system is a very effective tool in wheat marker development. Moreover, comparative genetic studies have demonstrated that gene content and orders are highly conserved, both at the map and megabase level, between different species within the grass family (Gale and Devos 1998a, 1998b), and hence PLUG is an effective marker developing system not only for wheat but also for other Triticeae species in which plenty of EST information has been obtained but little genomic sequence information is available. Besides, PLUG markers can specifically identify BAC clones containing homoeologous regions from the wheat A, B and D genomes (Ishikawa et al. 2009), and also can be efficiently transferred to other Triticeae species (Tsuchida et al. 2008; Li et al. 2013). Therefore, combining with the present low-cost and convenient separation method, namely microchip-based capillary electrophoresis (Liu et al. 2006; Kailasa et al. 2009), the PLUG system will definitely become a predominant tool to develop unique EST-PCR markers. Those markers can be used as accurate anchor markers for mapping, genome analysis, and comparative studies not only within hexaploid wheat chromosomes, but also between the genomes of rice, wheat and other grasses (Ishikawa et al. 2007, 2009).

For rye, Tsuchida et al. (2008) and Li et al. (2013) have developed 95 rye specific PLUG markers by transferring from the wheat PLUG markers, and have mapped these markers to individual rye chromosome arms. This fact demonstrated the high transferability of PLUG markers between different species in Triticeae.

The scope of this study

In summary, I would like to point out that in spite of the rapid progress in molecular and cytogenetics of rye over the past decades, the development of rye genomic analysis has lagged behind that of the other two species of the same tribe, wheat and

barley. Such status is chiefly caused by two inadequate research aspects, i.e. the lack of rye dissection lines and insufficient number of molecular markers, especially PCR-based markers. Therefore, considering these problems, this study was carried out with the following objectives:

1. Development of many PCR-based markers (PLUG markers) specific to the individual rye chromosome arms.
2. Detailed investigation of the syntenic relationships between the wheat and rye genomes through comparative genetic analysis.
3. Establishment of dissection lines for rye chromosomes in common wheat by the gametocidal system.
4. Test of the validity of a mass of rye SSR markers (or primer sets) and their assignment to the respective rye chromosomes.

CHAPTER 1

Development of PCR-based PLUG markers specific to individual rye chromosome arms

Abstract Based on the similarity in gene structure between rice and wheat, the PCR-based Landmark Unique Gene (PLUG) system enabled us to design primer sets that amplify wheat genic sequences including introns. From the previously reported wheat PLUG markers, I chose 144 markers that are distributed on different chromosomes and in known chromosomal regions (bins) to obtain rye-specific PCR-based markers. I conducted PCR with the 144 primer sets and the template of the Imperial rye genomic DNA and found that 131 (91.0%) primer sets successfully amplified PCR products. Of the 131 PLUG markers, 110 (76.4%) markers showed rye-specific PCR amplification with or without restriction enzyme digestion. I assigned 79 of the 110 markers to seven rye chromosomes (1R to 7R) using seven wheat-rye (cv. Imperial) chromosome addition and substitution lines: 12 to 1R, 8 to 2R, 11 to 3R, 8 to 4R, 16 to 5R, 12 to 6R and 12 to 7R. Furthermore, I located their positions on the short (S) or long (L) chromosome arm, using 13 Imperial rye telosomic lines of common wheat (except for 3RL). Referring to the chromosome bin locations of the 79 PLUG markers in wheat, I deduced the syntenic relationships between rye and wheat chromosomes. I also discussed chromosomal rearrangements in the rye genome with reference to the cytologically visible chromosomal gaps.

1.1 Introduction

Rye (*Secale cereale* L.) is a temperate cereal belonging to the tribe Triticeae, which is grown mainly in Europe, Northern America and across Asia. Its main uses include grain, hay, pasture, cover crop, green fodder, and green manure (Bartoš et al. 2008). Due to its broad tolerance to biotic and abiotic stresses, rye has an excellent tolerance to low temperatures and the ability to achieve relatively high yield under

environmental conditions in which other crops perform poorly (Madej 1996). Rye also plays an important role as a genetic source for wheat improvement, and it is a donor of the R genome to a man-made species triticale (x *Triticosecale* Wittmack) (Milczarski et al. 2011). In addition, rye is widely used by wheat breeders mainly because it is a precious gene pool reservoir, such as resistance genes to leaf rust (*Lr26*), stem rust (*Sr31*), stripe rust (*yr9*) and powdery mildew (*pm8*) (McIntosh 1988; Baum and Appels 1991).

Polymerase chain reaction (PCR) markers are easy to use to achieve much higher throughput analysis in the selection of DNA markers. Consequently, PCR-based markers have become one of the main tools for genetic analysis in wheat (Ishikawa et al. 2007). Various PCR-based markers, such as AFLP, SSR, RAPD, STS, SCAR, and EST have been used to construct genetic and cytological chromosome maps of rye (Philipp et al. 1994; Wanous and Gustafson 1995; Loarce et al. 1996; Korzun et al. 1998, 2001; Ma et al. 2001; Masojć et al. 2001; Bednarek et al. 2003; Bolibok et al. 2007; Milczarski et al. 2007; Hackauf et al. 2009; Gustafon et al. 2009; Gyawali et al. 2009, 2010; Stojałowski et al. 2009; Tenhola-Roininen et al. 2011; Tyrka et al. 2011). However, the practical application of those markers to rye chromosome mapping is greatly limited due to the small number of markers and consequently poor map density, the laborious and complicated technology employed, and the anonymous nature of the markers (Milczarski et al. 2011).

The development of molecular biology in Triticeae species has been hampered by their large genome sizes and insufficient genome sequence information. Despite a wide variation in genome size, all grass species show an extensive conservation of gene order along chromosomal segments (Gale and Devos 1998a). This so-called synteny reflects their descent from a common ancestral species approximately 50 million years ago (Paterson et al. 2004). Synteny can be exploited for map-based cloning in Triticeae (Krattinger et al. 2009).

The PLUG marker system developed by Ishikawa et al. (2007) is an effective tool for large-scale marker mining in wheat. We can design a pair of primers within separate exons of an EST, which can be inferred from the genomic sequences of rice

in the RAP database for which intron-exon structures are indicated (Rice Annotation Project 2008). Since the PLUG marker system is based on the conservation of orthologous genes, markers produced by this system can be used as accurate anchor markers for chromosome mapping, genome research, and comparative studies not only within wheat chromosomes but also among the genomes of rice, wheat and other grasses (Ishikawa et al. 2007, 2009). In fact, chromosome bin mapping of a set of PLUG markers located on the three homoeologous chromosomes of wheat (i.e. 1A, 1B, 1D), indicated that synteny among wheat homoeologous chromosomes is highly conserved except for the species-specific 4A-5A-7B translocations (Ishikawa et al. 2009).

In this study, I used wheat PLUG markers reported by Ishikawa et al. (2009) to develop rye-specific PCR-based markers, supposing that there should be divergence in the intron sequences of orthologous genes between rye and wheat. I selected representative PLUG markers mapped to the bins of the three wheat homoeologous chromosomes, expecting that they would allow the detection of the chromosomal rearrangements in the rye genome (Devos et al. 1993a; Martis et al. 2012). Moreover, I developed a few wheat-rye telocentric lines, which were previously unavailable, in this study and used an almost complete set of wheat-rye telocentric lines to assign the obtained rye-specific markers to each of the rye chromosome arms. This enabled us to examine the synteny break points (SBP; Murat et al. 2010), which would reveal the mode of karyotype evolution (Schubert 2007). I found novel six rearrangements in the rye genome and made a revision of the previously-proposed syntenic relationship between rye and wheat (Devos et al. 1993a; Martis et al. 2012).

1.2 Materials and Methods

1.2.1 Plant materials

I used wheat-rye chromosome addition and substitution lines that have individual chromosomes (1R to 7R) or chromosome arms (1RS to 7RL: S stands for the short arm and L for the long arm) of a rye cultivar Imperial (Imp) (*Secale cereale* L., 2n =

2x = 14, genome formula RR) in the genomic background of a common wheat cultivar Chinese Spring (CS) (*Triticum aestivum* L., 2n = 6x = 42, genome formula AABBDD). I obtained most of the above lines from Dr. Adam J. Lukaszewski, University of California, USA and John Innes Centre, Norwich, UK., and the rest (telocentric lines for 2RS, 5RS and 6RS) were developed in this study by screening the progeny of the monosomic addition plants for the respective chromosomes (Fig. 1-1, Supplementary Table S1-1, Supplementary Fig. S1-1). The 3RL telocentric line

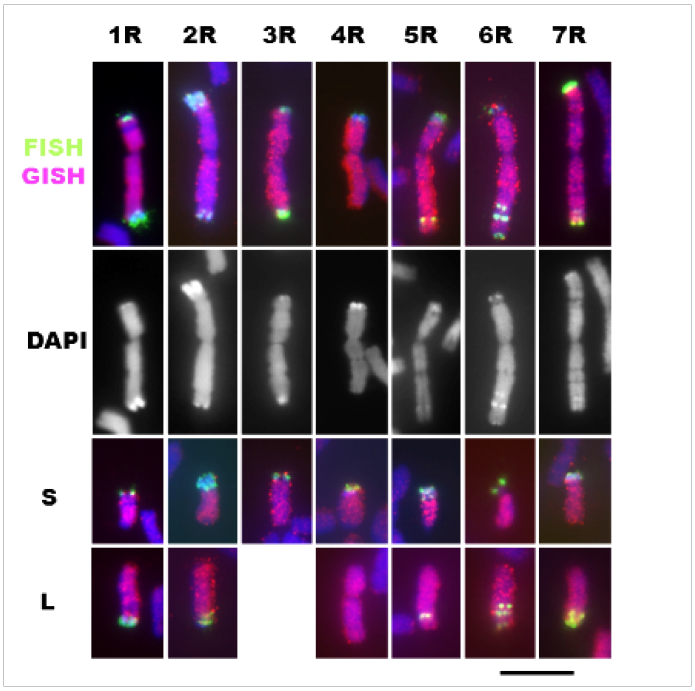


Fig. 1-1 FISH (*green*)/GISH (*pink*) and DAPI-stained photomicrographs of Imperial rye chromosomes and chromosome arms (*S*: short arm, *L*: long arm) in the wheat-rye addition and substitution lines. FISH probe: pSc200, GISH probe: rye total genomic DNA. The FISH/GISH and DAPI images are of the same chromosomes. Note that the gaps in chromosomes 2R to 7R are clearly visible in DAPI images. The sizes of the chromosomes and chromosome arms do not reflect their relative sizes in a cell because they are taken from different cells. *Bar*=10 μm.

was not available in this study. I cytologically confirmed that the 3R line was a disomic substitution for a wheat chromosome 3D (2n=42) (Supplementary Fig. S1-1). I also used euploid CS and Imp that have been maintained in our laboratory.

1.2.2 PLUG markers

I chose 144 primer sets from the 154 wheat PLUG markers (primer sets) that are localized in various bins of the 21 wheat chromosomes (Ishikawa et al. 2009) excluding some redundant markers in single bins for the present study (Table 1-1 and Supplementary Table S1-2). I extracted genomic DNA for PCR from fresh or frozen

Table 1-1. Assignment of the homoeologous group-1 PLUG markers to rye chromosome arms

PLUG[a] marker	PCR Primer		Rye chrom. arm assigned
	Forward	Reverse	
TNAC1009	CGAACGTGACCATCTACATCA	CATCTGACTTGGTCTTGGCATA	1RS
TNAC1019	AACGTGTCCACCGTCTACATC	CCAGTGGTCTCTGATTCATCC	1RS
TNAC1063	AGCCATTCACAGCTCTTCTTG	AATATGCTTCCTGGAGTCACG	1RS[b]
TNAC1021	CTCATGCATGCGTTTGTTAAA	CCAGCTGAAACAAGCATCTTC	1RL
TNAC1026	GGGATAGAACTCTGGGACTTCA	AGTGCCAGGGCATAATACAGC	1RL
TNAC1035	TGCACTGGGATCTAACCTAAA	TCCAGTGATCATTTGAAGATTCC	1RL
TNAC1041	TCACCACCTCTTTCAGTTGCT	GCATCAAGGATGAGGAGTCTG	1RL
TNAC1048	ACTGAGGTAGAATCGCCACTG	GCCGCTATCGTCTGGTACAT	1RL[b]
TNAC1052	TCAGCTGTGTCTGTTCTGTCCT	TGAGTCGACAAGACCACTCCT	1RL
TNAC1076	GGGAGACGATCCTCTTATGATCT	TGCGTGCTTCCTAACTTACCA	1RL
TNAC1086	CCAAACAAGATTGGATCATGG	CTGAGACCGTGCCCAGTG	1RL
TNAC1091	CTCTTCTCCTTGGGACCTTTG	GAACAGGACCTGCTGTTTGG	1RL[b]

[a] Name of PLUG marker cited from Ishikawa et al. (2009).

[b] Markers TNAC1063, TNAC1048, and TNAC1091 were miss-assigned to chromosme arms by Tsuchida et al. (2008), and corrected in the present study.

young leaf tissue either by the CTAB method (Saghai-Maroof et al. 1984) or by using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Each of the 25-μL PCR mixtures contained 50-100 ng of DNA, 1 μL of each primer set (10 pmol/μL), 1.5 μL of $MgCl_2$ (25mM), 0.5 μL of dNTP (10 mM), 5 μL of 5x PCR buffer, and 0.15 μL of Kapa Taq (5U/μL, KAPABIOSYSTEMS, USA). The PCR cycle consisted of an initial 5 min denaturation at 95°C, followed by 32 cycles of 95°C for 30 sec, 53-63°C (optimum 58°C) for 30 sec, and 72°C for 2 min, followed by a final extension at 72°C for 7 min, conducted with the iCycler (BioRad, USA). I analyzed a 5-μL aliquot of the amplification product by electrophoresis on a 1% agarose gel in 40 mM Tris-acetate, 1 mM EDTA (TAE) buffer at 100V for 30 min. For PCR-RFLP analysis, I digested an 8-μL aliquot of the product overnight with 5.0 U each of *Hae*III (at 37°C) and *Taq*I (at 65°C), and then fractionated the digested fragments by electrophoresis on a 4% agarose gel in TAE buffer at 100V for 50 min. I

stained the gel in a 0.5 mg/L ethidium bromide solution, for 30 min for the direct PCR products and for 1 h for the digested PCR fragments.

1.2.3 Cytology

I confirmed the presence of rye whole and telocentric chromosomes in the wheat-rye addition and substitution lines by FISH using the pSc200 sequences specific to the rye subtelomeres (Vershinin et al. 1995, 1996) and GISH using the total genomic DNA of Imp. I labeled the FISH probe with digoxigenin-11-dUTP (Roche, Germany) and the GISH probe with biotin-high prime labeling kit (Roche, Germany). I followed the FISH/GISH procedures described by Sakai et al. (2009) and Endo (2011). I recorded chromosome images with a digital camera (OLYMPUS DP80) set on a fluorescence microscope (OLYMPUS BX61).

1.3 Results

1.3.1 Selection of rye-specific PLUG markers

Using Imp genomic DNA as a template, I successfully obtained PCR products with 131 (91.0%) of the 144 PLUG primer sets: 115 primer sets amplified single bands of PCR products and 16 amplified two to four bands. PCR products could not be obtained with 13 primer sets (9.0%), at any annealing temperature. The direct PCR products of 18 (13.7%) of the 131 markers showed polymorphism between CS and Imp, and the PCR-RFLP analysis of additional 92 markers showed polymorphism between CS and Imp: 71 by *Taq*I digestion and 61 by *Hae*III digestion (40 by either restriction digestion). Thus, I obtained a total of 110 PLUG markers that can be used as specific markers for the rye genome.

1.3.2 Assignment of the rye-specific PLUG markers to rye chromosomes and chromosome arms

Using the wheat-rye chromosome addition and substitution lines, I sorted the 110 PLUG markers into seven chromosomes. When the rye-specific PCR product or

rye-unique restriction pattern of a PLUG marker occurred only in one rye addition and substitution line (Fig. 1-2), I assigned the PLUG marker to the critical rye chromosome. Thereby I successfully assigned 79 PLUG markers to individual rye chromosomes: 12 to 1R, 8 to 2R, 11 to 3R, 8 to 4R, 16 to 5R, 12 to 6R, and 12 to 7R (Table 1-1 and Supplementary Table S1-2). I failed in assigning the remaining 31 PLUG markers to any rye chromosomes due to unexpected PCR results, i.e., the absence of Imp-specific PCR products or restriction patterns in all wheat-rye addition and substitution lines.

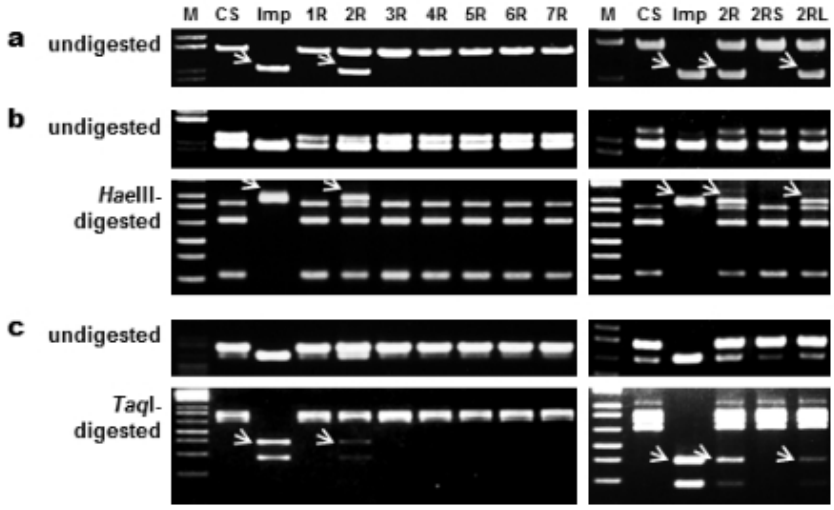


Fig. 1-2. Examples of PCR analysis to assign rye-specific PLUG markers to specific rye chromosomes and chromosome arms. *M*: 1kb DNA plus marker ladder, *CS*: Chinese Spring wheat, *Imp*: Imperial rye, *1R-7R*: wheat-rye chromosome addition and substitution (for 3R) lines, *2RS* and *2RL*: telosomic addition lines for the short arm and long arm of chromosome 2R, respectively. Note that all three PLUG markers (a: TNAC1142, b: TNAC1118, c: TNAC1200) showed rye-specific PCR amplifications (indicated with arrows) only in Imp, the 2R and 2RL addition lines in the undigested PCR product (a) or after restriction enzyme digestion (b, c).

I used the thirteen wheat-rye telosome addition lines to allocate the 79 PLUG markers to the short or long arm of the critical rye chromosome. As shown in Table 1, Supplementary Table S1-2 and Supplementary Fig. S1-2, I successfully allocated all the 79 markers to the respective long or short arm: all markers showed their rye chromosome-specific PCR amplification in either of the two telocentric lines of the corresponding rye chromosome. Since 3RL telocentric line was not available in this

study, I assigned the 3R-specific markers that showed no PCR amplification in the 3RS telocentric line to the 3RL arm.

1.3.3 Comparison of the locations of the PLUG markers on the rye and wheat chromosomes

I compared the bin-locations of the 79 PLUG markers on the wheat chromosomes (Ishikawa et al. 2009) with the arm-locations of the same markers on the rye chromosomes and constructed comparative maps for individual rye chromosomes (Fig. 1-3). For chromosome 1R (Table 1-1), all three 1RS markers were derived from

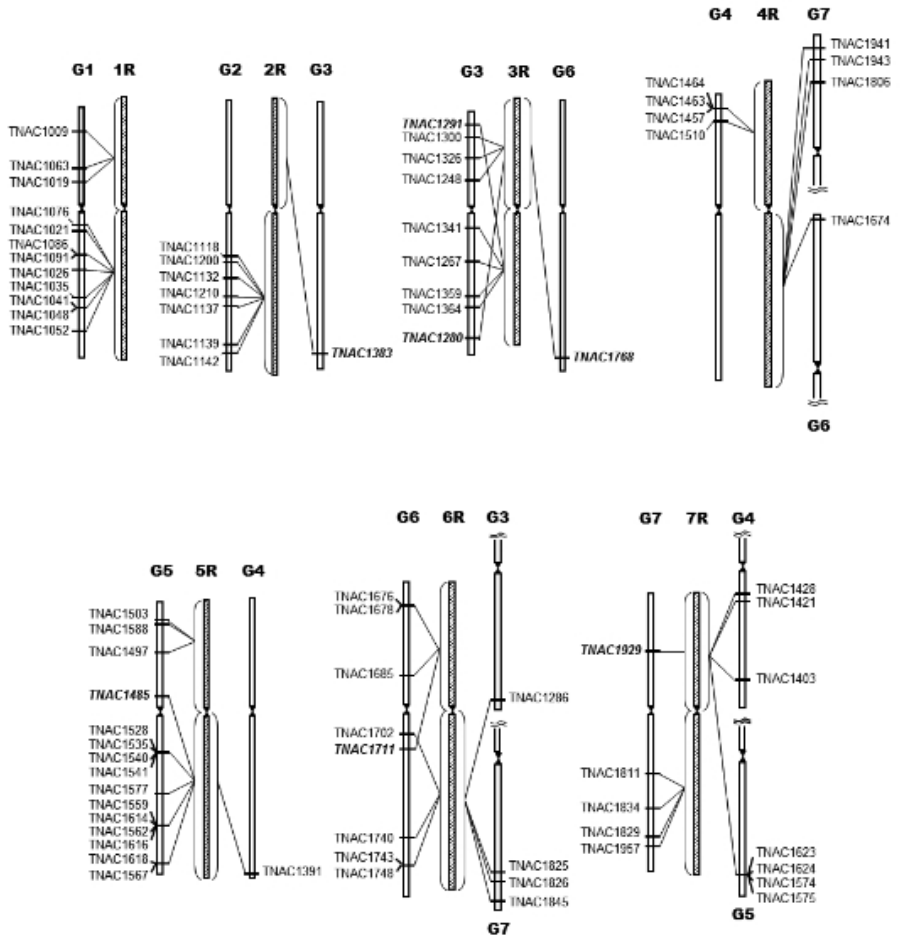


Fig. 1-3. Comparative chromosome mapping of PLUG markers between wheat and rye. The locations of the 79 PLUG markers have been determined by chromosome bin mapping (Ishikawa et al. 2009). For every chromosome map, chromosomes are orientated as the short arm in the *upper* and the long arm in the *lower part* of the scheme. *G*: wheat homoeologous group, *R*: rye chromosome. See the text for the details of homoeologous relationships (indicated with the *markers* in italics) reported for the first time in this study.

the short arm of the wheat homoeologous group 1 (1S); all nine 1RL markers corresponded to those on the long arm of the wheat homoeologous group 1 (1L). For chromosome 2R, one 2RS marker was derived from the wheat 3L arm; all seven 2RL markers were derived from the wheat 2L arm. For chromosome 3R, three of the 3RS markers were derived from the wheat 3S arm, whereas one was from the wheat 3L arm and one was from the wheat 6L arm; four of the 3RL markers were derived from the wheat 3L arm, while one was from the wheat 3S arm. For chromosome 4R, all four 4RS markers were derived from the wheat 4S arm; three of the 4RL markers were derived from the wheat 7S arm, and one was from the wheat 6S arm. For chromosome 5R, all three 5RS markers were derived from the wheat 5S arm; 11 of the 5RL markers were derived from the wheat 5L arm, while one was from the wheat 4L arm, and one was from the wheat 5S arm. For chromosome 6R, three of the 6RS markers were derived from the wheat 6S arm, whereas one was from the wheat 6L arm; four of the 6RL markers were derived from the wheat 6L arm, while one was from the wheat 3L arm, and three were from the wheat 7L arm. For chromosome 7R, one of the 7RS markers was derived from the wheat 7S arm, while three were from the wheat 4L arm and four were from the wheat 5L arm; all four 7RL markers were derived from the wheat 7L arm.

1.3.4 Confirmation of translocation involving the long arm of homoeologous group 3 chromosomes

I detected one 2RS marker (TNAC1383) derived from the most distal bins in wheat (3AL8-0.85-1.00; 3BL11-0.81-1.00; 3DL3-0.81-1.00; Ishikawa et al. 2009). I found another marker TNAC1286 localized to the same bins as TNAC1383 that was mapped to chromosome 6R. The marker TNAC1280 that is mapped to the next proximal bins in wheat (3AL5-0.79-0.85; 3BL7-0.63-0.81; 3DL1-0.23-0.81) was located on the 3R chromosome. The indicated multiple chromosome rearrangements involving the most distal part of ancestral 3RL were tested by 10 PLUG markers mapped to the most distal bin on the group-3 chromosomes in wheat (Ishikawa et al.

2009; Saito et al., unpublished data), and seven PLUG markers that were deduced to localize in physical proximity of those markers based on GenomeZipper (Mayer et al. 2011; Saito et al., unpublished data). I could obtain rye-specific amplification in five markers; TNAC3773 was assigned to chromosome arm 3RS, and TNAC3809 and TNAC3817 to 6RL, TNAC1382 to 4RL, and TNAC1384 to 3RL. Thus, the translocation involving chromosome 2R was not verified by additional markers.

1.4 Discussion

PCR-based markers have been widely used in genetic analysis (Cox and Lehrach 1991) because they can be checked with a small amount of DNA and without the time-consuming blotting procedure required for RFLP markers (Konieczny et al. 1993). Recently, large volumes of single nucleotide polymorphisms (SNPs) were searched by sequencing of known genes (Li et al. 2011) and RNA-seq approaches (Haseneyer et al. 2011). However, those SNPs have not been genetically mapped to rye chromosomes. Although a consensus genetic map of rye harboring a total of 9703 segregating markers was constructed using a microarray platform (Milczarski et al. 2011), the homology of the markers to ESTs of other grass species, especially to wheat and barley ESTs, remains unknown.

In addition to cytological methods such as chromosome banding and *in situ* hybridization techniques, PCR-based markers have also become a convenient and efficient tool to identify the presence of alien chromosomes and chromosomal segments in wheat. Since Koebner (1995) first generated PCR-based markers for the detection of rye chromatin in the wheat background, many PCR-based markers have been developed for the studies of rye genetics and breeding programs (Saal and Wricke 1999; Brunell et al. 1999; Nagy and Lelley 2003; Isik et al. 2007; Kofler et al. 2008; Lee et al. 2009; Wang et al. 2009). However, such markers are mostly specific to the rye genome in general, and only a few of them are specific to individual chromosomes, e.g., chromosomes 1R and 2R (Brunell et al. 1999; Nagy et al. 2003; Kofler et al. 2008; Lee et al. 2009; Wang et al. 2009), or to the organellar genomes

(Isik et al. 2007). Saal and Wricke (1999) developed 27 rye-specific SSR markers and mapped 20 of them onto individual chromosomes or chromosome arms, and other researchers developed some more PCR-based markers specific to rye chromosomes in the past few years (Varshney et al. 2005; Kofler et al. 2008; Lee et al. 2009; Wang et al. 2009; Xu et al. 2012), however, the number of markers is far from sufficient for the genomic studies of rye.

PLUG markers are based on the sequence conservation of orthologous genes, and therefore their transferability between Triticeae species should be high. In this study, I obtained 110 markers polymorphic between CS and Imp out of the 144 wheat PLUG markers and successfully assigned 79 (55%) of them to individual rye chromosome arms (Table 1-1 and Supplementary Table S1-2). With the same success rate, I could have obtained close to 300 rye chromosome-specific markers if I had used all 531 wheat PLUG markers developed by Ishikawa et al. (2009). Since Ishikawa et al. (2007) identified over 4,000 candidate LUGs (landmark unique genes) in wheat ESTs, I would be able to develop more PLUG markers. Moreover, owing to recent advances in barley genomics I can map the PLUG markers to GenomeZipper (Mayer et al. 2011) and barley physical maps (The international barley genome sequencing consortium, 2012). Besides, I will be able to design rye PLUG markers directly from rye EST databases. Recently, Xu et al. (2012) developed 31 rye chromosome-specific EST-based markers by screening a total of 1191 primer pairs derived from wheat and rye EST sequences at a very low success rate (3%). This fact indicates the superlative efficiency of the PLUG marker system in developing species-specific PCR-based markers.

Although the transferability of the PLUG markers from the wheat genomes to the rye genome was very high (55%), I still could not assign 31 of the 110 rye-specific markers to any of the rye chromosomes by using the wheat-rye addition and substitution lines: the PCR patterns of the 31 markers that appeared in Imperial rye did not appear in any of the wheat-rye addition and substitution lines (Fig. 1-4). This oddity might be due to the genetic polymorphism between the Imperial rye used as the control in this study and the one used for the development of the wheat-rye

addition lines. In this connection, Alkhimova et al. (1999) reported cytological polymorphism within Imperial rye, as well as among different rye cultivars. Tsuchida et al. (2008) had assigned the same three 1R-specific markers as I used in this study to different chromosome arms: TNAC1048 to 1RS, and TNAC1063 and TNAC1091 to both arms. I repeated PCR analysis with the three markers, using different sources of the 1RS and 1RL ditelosomic lines to confirm their arm locations as shown in Table 1-1 and Supplementary Table S1-2.

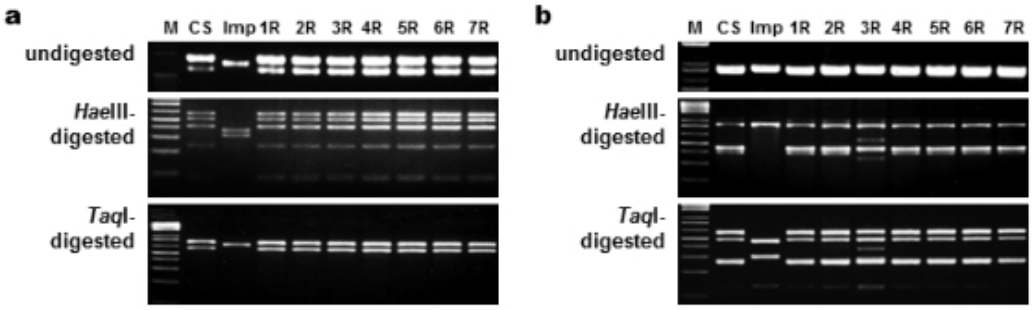


Fig. 1-4. Examples of uncertain results of PCR analysis. *M*: 1kb DNA plus marker ladder, *CS*: Chinese Spring wheat, *Imp*: Imperial rye, *1R*-*7R*: wheat-rye chromosome addition and substitution (for 3R) lines. Note we could amplify PLUG markers (a) TNAC1140 and (b) TNAC1356 in Imp but not in any of the wheat-rye addition and substitution lines.

I found nearly the same syntenic relationships between the rye and wheat chromosomes as were previously reported (Devos et al. 1993a; Martis et al. 2012). However, the combination of bin-mapped wheat markers and the set of rye telosiomic addition and substitution lines allowed detection of synteny break points within a given rye chromosome. The chromosomal locations of seven markers (TNAC1383, TNAC1280, TNAC1768, TNAC1291, TNAC1485, TNAC1711 and TNAC1929; indicated in bold italics in Fig. 1-3) suggested revisions of the previous syntenic relationships proposed by Devos et al. (1993a) and Martis et al. (2012). Most of the PLUG markers on the 3RS and 3RL arms originated from the wheat 3S and 3L arms, respectively, but the 3RS arm carried two markers TNAC1280 and TNAC1768 derived from the wheat 3L and 6L arms, respectively and that the 3RL arm carried TNAC1291 located on the wheat 3S arm. Therefore, to identify the structure of the present 3R chromosome, I need to presume that an interchromosomal translocation

occurred between the ancestral 3R and 6R chromosomes, followed by an inversion within the translocated 3R chromosome, which was indicated by EST mapping by Qi et al. (2006). The other four PLUG markers on different rye chromosomes also showed complex syntenic relationships between the wheat and rye genomes that had not been known in the previous studies. Marker TNAC1383 located on the wheat 3L arm was assigned to the 2RS arm, marker TNAC1485 on the wheat 5S arm was assigned to the 5RL arm, marker TNAC1711 on the wheat 6L arm was assigned to the 6RS arm, and marker TNAC1929 on the wheat 7S arm was assigned to the 7RS arm. From these syntenic relationships of the markers, I deduced chromosomal rearrangements that most likely occurred during the karyotype evolution of rye (Fig. 1-5). When the syntenic relationships are reviewed from the point of homoeologous chromosomes in wheat, I noticed that the most distal part of the long arm of group-3 (G3) chromosome seems to have been involved in multiple translocations among chromosomes 2R, 3R, and 6R. Additional tests of the markers located on the distal bins in wheat G3 chromosomes verified the 3R/6R translocation occurring between markers TNAC3773 and TNAC1286 whose rice counterparts, Os01g0928100 and Os01g0963300 respectively, are 1.7 Mb apart on rice chromosome 1 (RAP-DB, Rice Annotation Project (2008)) and 27 Mb apart on the barley hypothetical physical map (International Barley Genome Sequencing Consortium 2012). I could not confirm the 2R/3R translocation by other markers located in the vicinity of TNAC1383. Thus, it is possible that the 2R-localization of the marker TNAC1383 reflects single-gene movement frequently found in large and complex Triticeae genomes (Wicker et al. 2011). Other singlet marker TNAC1382 localized to 4RL may be another case of such single-gene movement. The 3RL localization of TNAC1384, whose rice homologue is present in the most distal region of chromosome 1, could be explained by the pericentromeric inversion since the wheat and barley homologues are located on G3S chromosomes. By assigning markers to rye chromosome arms, I could show at least one pericentromeric inversion (chromosome 3R), and the discrepancy of arm localization (chromosomes 5R, 6R, and 7R) which might be explained by cryptic

pericentromeric inversion. Our findings support the findings by Devos et al. (1993a) and Martis et al. (2012) that rye karyotype changed more rapidly than wheat and

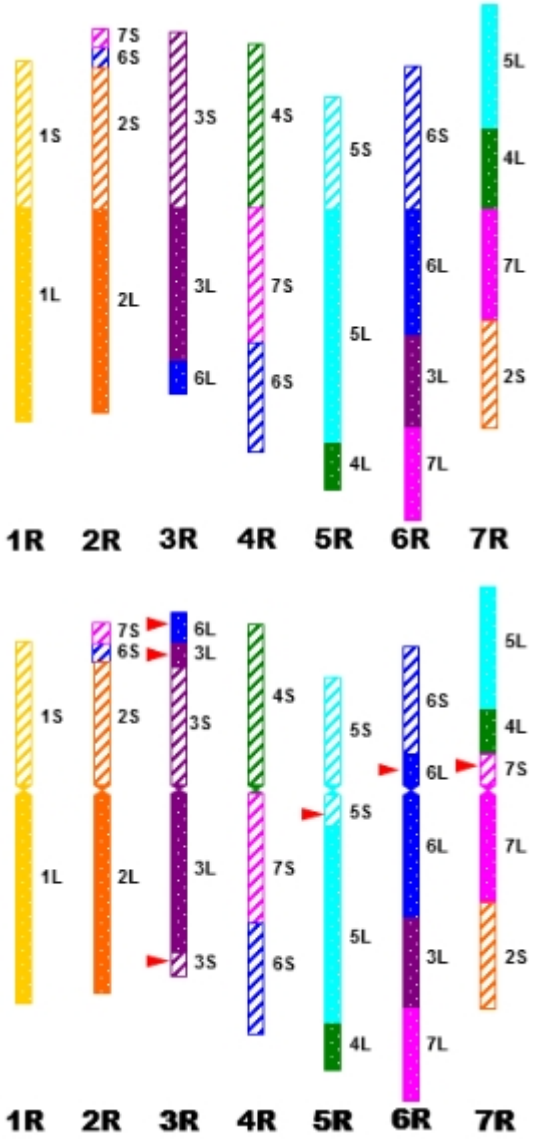


Fig. 1-5. Most likely synteny maps (the *lower set*) showing rearrangements of the rye chromosomes constructed by combining the data obtained in this study and those reported in previous studies (Devos et al. 1993a; Martis et al. 2012). The *upper set* of maps is cited from Fig. S8 of Martis et al. (2012). The *arrow heads* indicate positions of the novel syntenic relationships found in this study.

barley genomes. Careful observation of DAPI stained rye chromosomes revealed stably visible gaps (see DAPI images in Fig. 1-1), which may reflect structural rearrangements that might have occurred during evolution. Since the number of such gaps is more than the number of the breakpoints of chromosomal rearrangements

inferred from the present and previous studies (Fig. 1-5), the rye genome might have suffered from more complicated chromosomal rearrangements. I will be able to address the issue of a correspondence between the chromosomal gaps and rearrangements in rye by increasing the number of chromosome-specific markers and at the same time by chromosome microdissection (Zhou et al. 1999) or developing dissection lines of common wheat carrying rye chromosomal segments, as developed for chromosome 1R (Gyawali et al. 2009).

CHAPTER 2

Dissection of rye chromosomes by the gametocidal system

Abstract Chromosome mutations occur in common wheat carrying a monosome of gametocidal chromosomes 2C and $3C^{SAT}$. These gametocidal chromosomes also induce chromosomal breakage in a rye chromosome 1R added to common wheat. I attempted to introduce these gametocidal chromosomes into the other six rye chromosome (2R to 7R) addition or substitution lines of common wheat and succeeded in producing common wheat lines that were disomic for each of the rye chromosomes, except for 5R and 7R, and monosomic for either 2C or $3C^{SAT}$. In the selfed progeny of these lines, I established common wheat lines that were double-disomic for the rye chromosome (2R, 3R and 6R) and gametocidal chromosomes. During the production of these lines, I found structural changes such as deletions and translocations involving every rye chromosome. Thus I demonstrated that the gametocidal chromosomes were capable of inducing chromosome mutations in all the six rye chromosomes, as well as in 1R. Henceforth, it has been proved that we can produce dissection lines for every rye chromosome in common wheat by the gametocidal system.

2.1 Introduction

Rye (*Secale cereale* L., 2n=2x=14, RR) is a diploid outbreeding species, belonging to the tribe Triticeae (Poaceae), and it is grown primarily in Europe, and in parts of Northern America, South America and Asia. It is second to wheat among grains used in the production of bread and is also important for the production of mixed animal fodder (Bushuk 2001). Thanks to its excellent biotic and abiotic stress tolerance (Mohammadi et al. 2003; Bartoš et al. 2008), together with its superior dietary value (Isaksson et al. 2009), rye makes an important genetic resource in the breeding programs of wheat. However, due to the huge size (1C = 7917 Mbp) (Bartoš et al.

2008) and rearranged complex structure of the rye genome containing highly repetitive sequences DNA (about 70 to 75%) (Ranjekar et al. 1974; Appels et al. 1978; Hutchinson and Lonsdale 1982; Vershinin et al. 1995), it is a great challenge to order billions of short reads obtained with new sequencing techniques and reconstruct a complete rye genomic sequence (Pop and Salzberg 2008). In contrast to small-genome model species, where whole-genome shotgun sequencing is sufficient, physical maps are mandatory for the development of whole genome reference sequences of large and complex genomes, such as those of the Triticeae crop species, wheat, barley and rye (Stein 2009).

The construction of high-density chromosome maps is a prerequisite for gene cloning and molecular breeding in crops. Till now, cytological maps, the lowest-resolution physical chromosome maps, have been constructed in common wheat (*Triticum aestivum* L. 2n=6x=42, AABBDD) (Werner et al. 1992; Kota et al. 1993; Delaney et al. 1995a and 1995b; Mickelson-Young et al. 1995; Gill et al. 1996a and 1996b; Randhawa et al. 2004; Hossain 2004; Qi et al. 2004) by using a series of the deletion stocks of common wheat (Endo and Gill 1996). Cytological maps have also been constructed in barley (*Hordeum vulgare* L., 2n=2x=14, HH) by using a series of dissection lines of common wheat carrying barley deletion chromosomes or barley-wheat translocations (Shi et al. 1999; Ashida et al. 2007; Sakai et al. 2009; Sakata et al. 2010; Joshi et al. 2011). Both wheat stocks and lines were generated by the gametocidal system of chromosomes 2C from *Aegilops cylindrica* and $3C^{SAT}$ from *Aegilops triuncialis* (Endo 1990, 2007). Rye lags behind wheat and barley in genetic and genomic studies (Milczarski et al. 2011). Besides, cytological mapping of rye chromosomes has seldom been conducted, except for rye chromosome 1R (Masoudi-Nejad et al. 2002; Tsuchida et al. 2008; Gyawali et al. 2009, 2010). This is simply due to the lack of the dissection lines for rye chromosomes other than 1R. To accelerate genetic and genomic research in rye, therefore, we need produce dissection lines for all rye chromosomes.

In the present study, I applied the gametocidal system to the rye addition or substitution lines of common wheat in order to dissect rye chromosomes 2R to 7R in

common wheat. Here I report the breeding process of wheat-rye addition or substitution lines carrying the gametocidal chromosome, whose progeny includes plants with aberrant rye chromosomes such as deletions and wheat-rye translocations.

2.2 Materials and Methods

2.2.1 Plant materials and Crossing scheme

I used six rye addition and substitution lines of a common wheat cultivar Chinese Spring (CS) (*Triticum aestivum* L, 2n=6x=42, genome formula AABBDD) that were disomic for individual chromosomes (2R to 7R) of a rye cultivar Imperial (Imp) (*Secale cereale* L, 2n=2x=14, RR). I obtained all these lines from Dr. Adam J. Lukaszewski, University of California, USA. To dissect the rye chromosomes by the gametocidal (Gc) system, I used two Gc chromosomes, 2C from *Aegilops cylindrica* (2n=4x=28) (Endo 1988, 1990, 1996) and $3C^{SAT}$ from *Ae. triuncialis* (2n=4x=28) (Endo 1978, 1996). I also used the euploid Imp rye, *Aegilops triuncialis* (genome UUCC, accession KU6666) and *Ae. caudata* (genome CC, accession KU12033) for the probe DNA of in situ hybridization. The 2C and $3C^{SAT}$ disomic addition lines of CS, and the euploid lines of CS and Imp had been maintained in our laboratory, and the *Ae. triuncialis* and *Ae. caudate* accessions were obtained from National BioResource Project, Japan (NBRP-wheat).

Figure 2-1 shows the crossing scheme to produce common wheat lines that are disomic for the respective rye chromosomes and monosomic for the 2C or $3C^{SAT}$ gametocidal chromosome. Since we can obtain plant carrying chromosome mutations in the progeny, I will call these lines ‘chromosome-mutation inducing lines’ in the text.

2.2.2 Cytology

Chromosome preparation and *in situ* hybridization

I made chromosome spreads by the squash method using root tips that were immersed in ice-cold distilled water for ca. 20 h and fixed in 3:1 fixative for ca. one

week at room temperature, and stained in a 1% acetocarmine solution for 1-2 h. I conducted fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) as described by Sakai et al. (2009) and Endo (2011), using rye subtelomeric-specific pSc200 sequences (amplified by PCR) (Vershinin et al. 1995, 1996), and the total genomic DNA of rye, *Ae. triuncialis* and *Ae. caudata*.

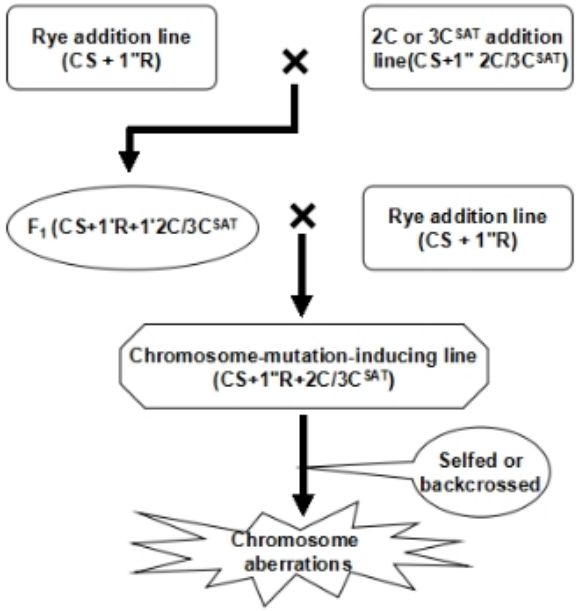


Fig. 2-1. Breeding scheme for the genetic induction of structural changes in rye chromosomes added to common wheat. CS, R and 2C/3C^{SAT} stand for Chinese Spring wheat, rye chromosomes, and chromosomes 2C or 3C^{SAT}, respectively.

2.3 RESULTS

2.3.1 Cytological examination of the backcrossed progeny of the hybrids between the rye addition/substitution and gametocidal lines

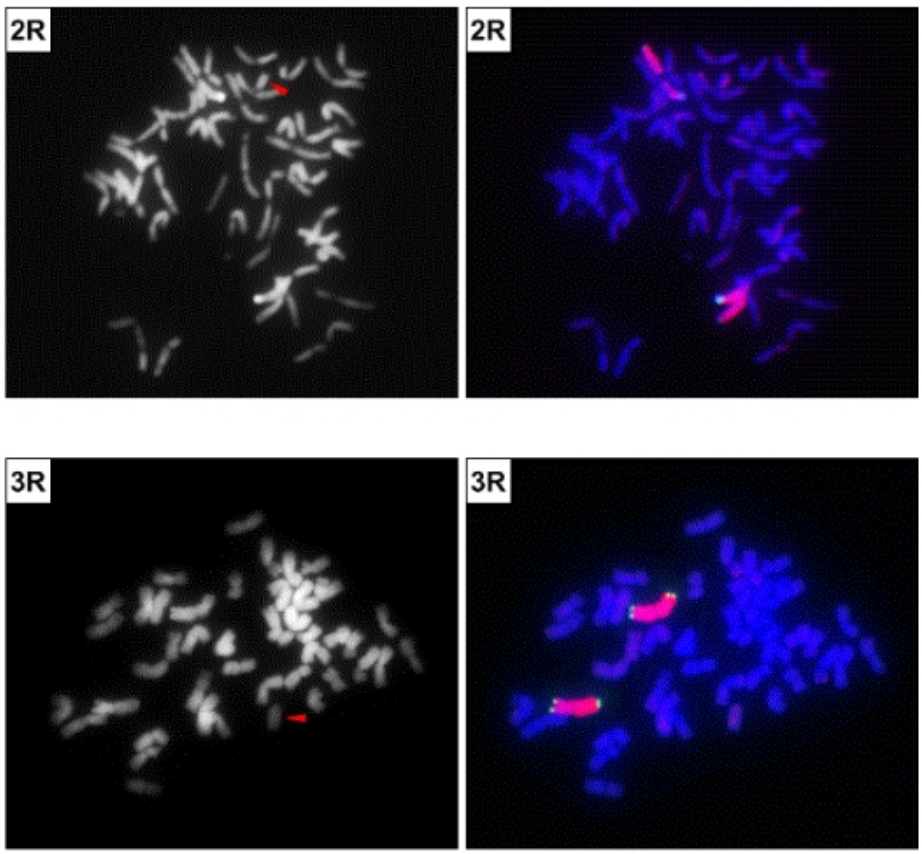
I crossed the five wheat-rye disomic addition lines (2n=44) and one substitution line (3R, 2n=42) as female to the 2C and 3C^{SAT} addition lines, with failing a cross between the 7R and 2C addition lines. I backcrossed the hybrids (2n=44, 2n=43 for 3R) to the respective parental rye addition or substitution lines to obtain BC_1 progeny. Since no disomic 5R addition plants were available at the time of crossing, I used monotelodisomic (5R/5RS) plants for backcrossing.

Table 2-1. Cytological screening of the backcrossed and selfed progeny of the double monosomic plants for the individual rye chromosomes and Gc chromosome

Backcross or self ♀ x ♂	seed set (%)	germination (%)	No. plants examined	No. plants disomic for the rye chromosome and monosomic for the Gc chromosome	No. plants with rye telocentric and aberrant chromosomes[1)]
2R' + 2C' × 2R"	45.0	100.0	72	5	6
3R' + 2C' × 3R"	66.3	100.0	40	4	1
4R' + 2C' × 4R"	52.5	97.6	41	0	4 (1)
5R' + 2C' × 5R'/5RL'	54.4	98.9	87	1[2)]	23 (3)
5R' + 2C' self	/	95.8	23	2[2)]	2 (1)
6R' + 2C' × 6R"	40.8	88.5	46	1	2 (telosome or del-)
7R' + 2C' × 7R"	/	/	/	/	/
2R' + $3C^{SAT'}$ × 2R"	54.7	97.9	96	2	10
3R' + $3C^{SAT'}$ x 3R"	80.8	100.0	36	3	1
4R' + $3C^{SAT'}$ x 4R"	68.9	97.9	95	2	0
5R' + $3C^{SAT'}$ x 5R'/5RL'	80.0	100.0	42	1[2)]	14
6R' + $3C^{SAT'}$ x 6R"	64.4	100.0	100	0	2
7R' + $3C^{SAT'}$ x 7R"	52.9	98.7	76	1[2)]	8

[1)] Those with aberrant rye chromosomes are in parentheses.

[2)] Monotelodisomic for the rye chromosome.



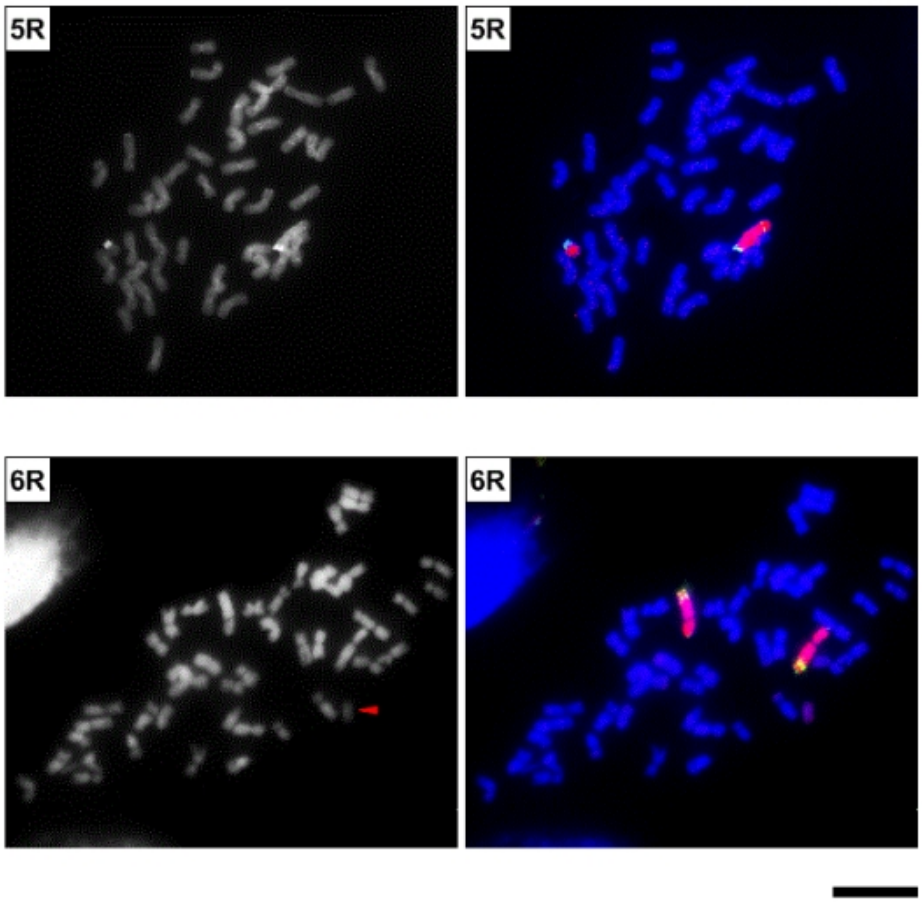


Fig. 2-2. DAPI (left) and FISH(green)/GISH(pink) (right) images of the mitotic metaphase cells of the 45-chromosome and 43-chromosome (for 3R) plants that are disomic for each of the rye chromosomes and monosomic for 2C or $3C^{SAT}$ (indicated with an arrowhead). Bar = 10 μm.

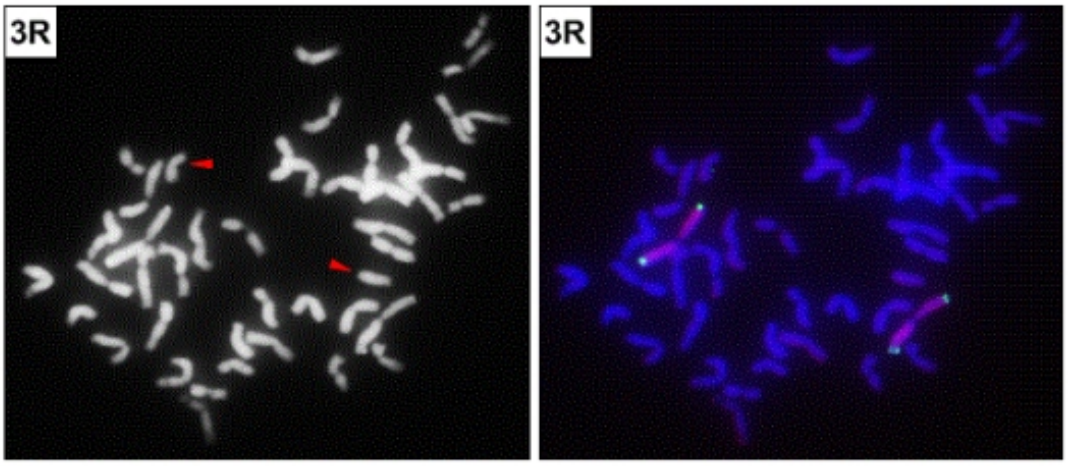
Screening the BC_1 progeny, I obtained critical plants carrying 2C for 2R (2n=45), 3R (2n=43) and 6R (2n=45) and those carrying $3C^{SAT}$ for 2R (2n=45), 3R (2n=43) and 4R (2n=45) (Table 2-1). These plants were disomic for each of the rye chromosomes and monosomic for 2C or $3C^{SAT}$ (Fig. 2-2). I failed in obtaining critical plants for the remaining chromosome combinations of the rye and gametocidal chromosomes. For 5R and 7R, I obtained semi-critical plants instead, i.e. monotelodisomic additions of one whole and one telosome of the rye chromosome, together with 2C or $3C^{SAT}$. During this screening I found many plants with rye telosomes, not to mention 5RS, and some with structural aberrations involving the rye chromosomes (Table 2-1).

2.3.2 Cytological examination of the BC_2 and BC_1F_2 progeny

I cytologically screened the BC_2 and BC_1F_2 progeny of the critical and semi-critical plants. I obtained more critical plants for 2R, 3R and 4R and alien double-disomic plants for the rye and the gametocidal chromosomes for 2R and $3C^{SAT}$, for 3R and 2C, and for 6R and 2C (Table 2-2, Fig. 2-3). I failed in obtaining neither critical plants nor double-disomic alien addition plants for 7R and $3C^{SAT}$.

Table 2-2. Cytological screening of the backcrossed and selfed progeny of the plants disomic for the individual rye chromosomes and monosomic for the Gc chromosome

Backcross or self ♀ x ♂	No. plants examined	No. plants disomic for the rye chromosome and monosomic for the Gc	No. plants disomic for the rye and Gc chromosomes	No. plants with aberrant rye chromosomes except telosomes
2R" + 2C' × 2R"	6	4	0	1
2R" + 2C' self	30	1	0	6
3R" + 2C self	201	32	3	35
5R'/5RL' + 2C self				
5R'/5RS' + 2C self				
6R" + 2C' × 6R"	17	5	0	1
6R" + 2C' self	37	9	5	2
2R" + $3C^{SAT'}$ × 2R"	9	0	0	2
2R" + $3C^{SAT'}$ self	135	2	1	20
4R" + $3C^{SAT'}$ × 4R"	13	0	0	0
4R" + $3C^{SAT'}$ self	30	5	0	1
5R'/5RL' + $3C^{SAT'}$ self	40	0	0	2
7R'/7Rtelo' + $3C^{SAT'}$ × 7R"	32	0	0	1
7R'/7Rtelo' + $3C^{SAT'}$ self	81	0	0	3



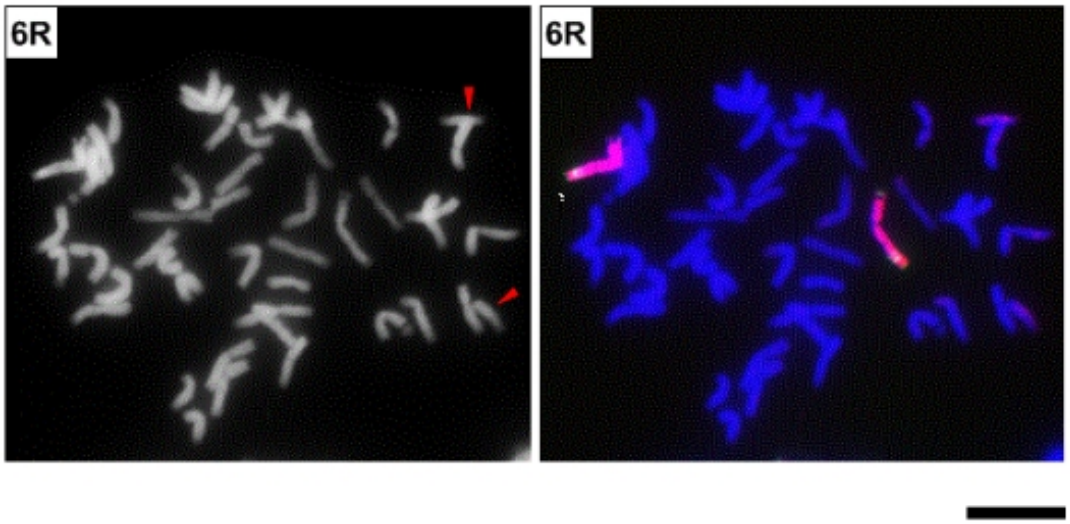
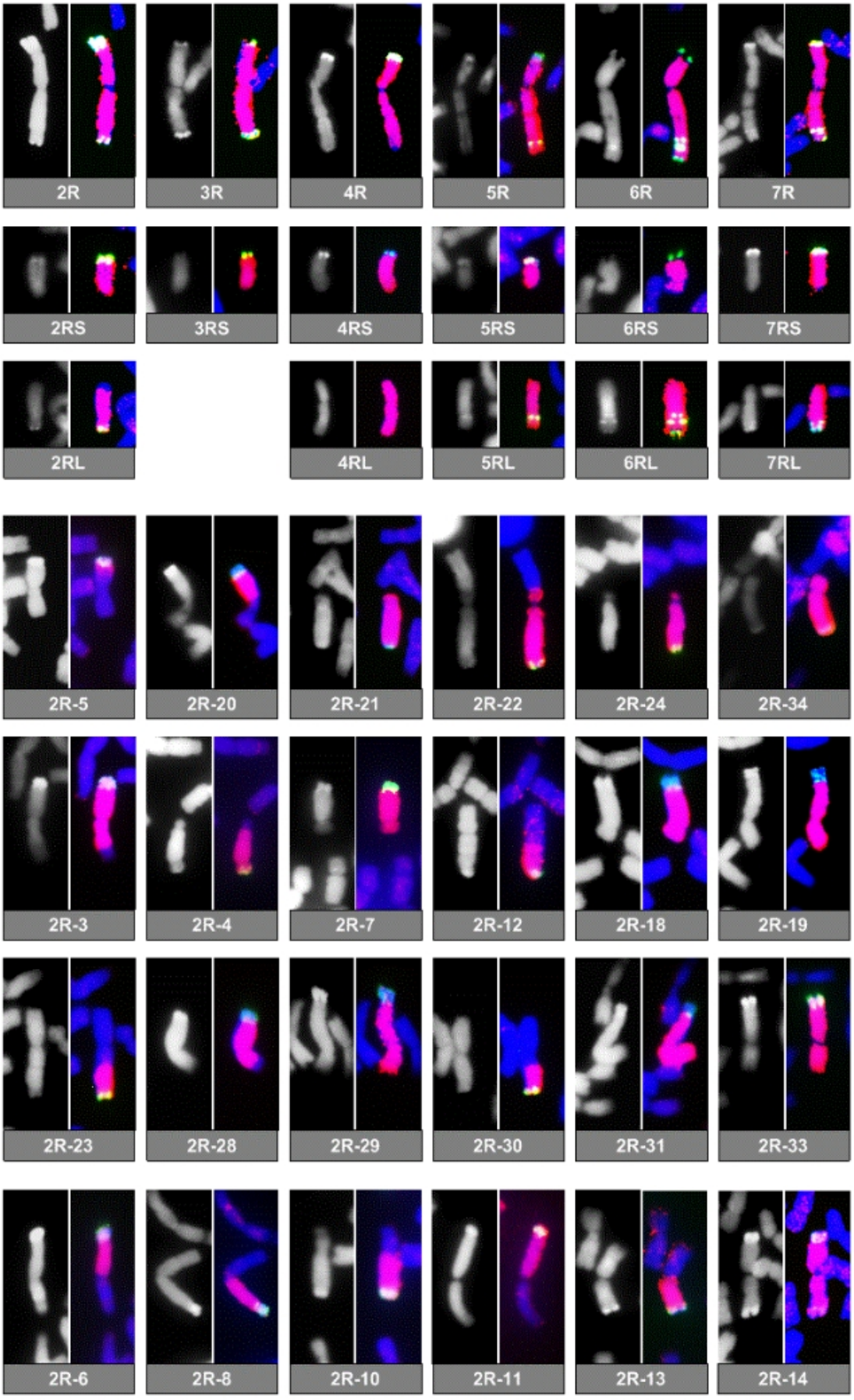


Fig. 2-3. DAPI (left) and FISH(green)/GISH(pink) mitotic metaphase of 46-chromosome and 44-chromosome (for 3R) plants that are double-disomic for each of the rye chromosomes and for 2C or $3C^{SAT}$ (indicated with two arrowheads). Bar = 10 μm.

2.3.3 Identification of aberrant rye chromosomes

During the screening of the above-mentioned backcrossed and self-pollinated progeny using FISH/GISH techniques, I identified various types of aberration in the rye chromosomes. Although I found many rye telosomes and isochromosomes, I do not include them in the aberrant rye chromosomes induced by the gametocidal action because I used monotelo-disomics in some crosses and because rye univalent chromosomes often suffer from centromeric misdivision to generate telosomes and isochromosomes. I found 29 aberrant chromosomes for 2R, 35 for 3R, 1 for 4R, 2 for 5R, 3 for 6R and 4 for 7R (Fig. 2-4, Table 2-2).

There were a total of 41 deletions excluding telosomes and 33 translocations. The 41 had breakpoints in either of the arms: 20 in the short arms (3 in 2RS, 16 in 3RS and 1 in 6RS), 19 in the long arms (6 in 2RL, 11 in 3RL, 1 in 5RL and 1 in 7RL), and 2 in both arms (2R-32 and 4R-3). I found three types of translocation among the 33 translocated chromosomes: 8 Robertsonian chromosomes, 14 wheat-to-rye translocations (rye chromosomes with wheat chromosomal segments), and 11 rye-to-wheat translocations (wheat chromosomes with rye chromosome segments). Among the 8 Robertsonian translocations 4 involved 2RS (2R-6, 2R-11, 2R-15 and


2R
3R
4R
5R
6R
7R
2RS
3RS
4RS
5RS
6RS
7RS
2RL
4RL
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7RL
2R-5
2R-20
2R-21
2R-22
2R-24
2R-34
2R-3
2R-4
2R-7
2R-12
2R-18
2R-19
2R-23
2R-28
2R-29
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2R-6
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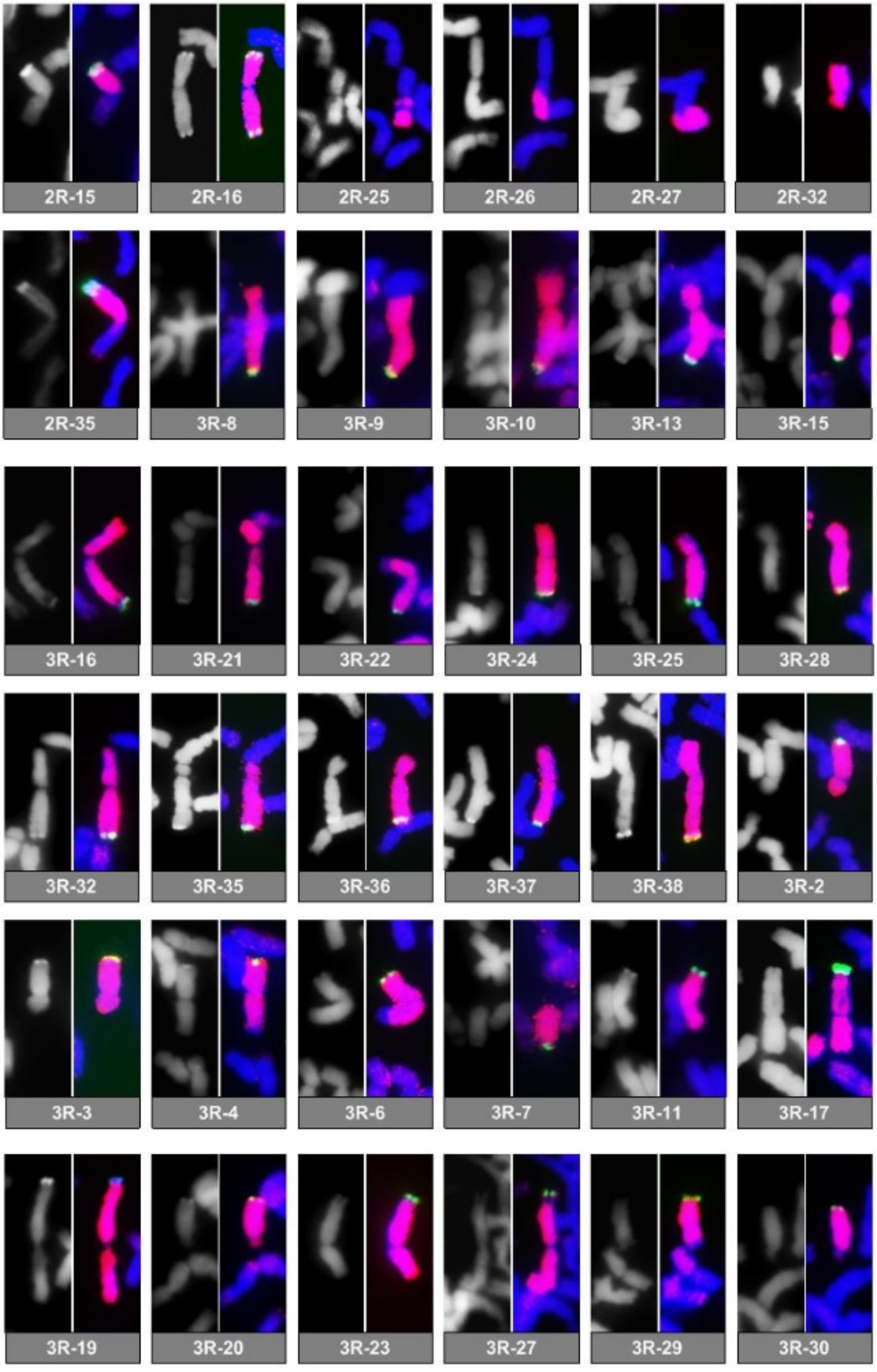
2R-11
2R-13
2R-14


2R-15
2R-16
2R-25
2R-26
2R-27
2R-32
2R-35
3R-8
3R-9
3R-10
3R-13
3R-15
3R-16
3R-21
3R-22
3R-24
3R-25
3R-28
3R-32
3R-35
3R-36
3R-37
3R-38
3R-2
3R-3
3R-4
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3R-27
3R-29
3R-30

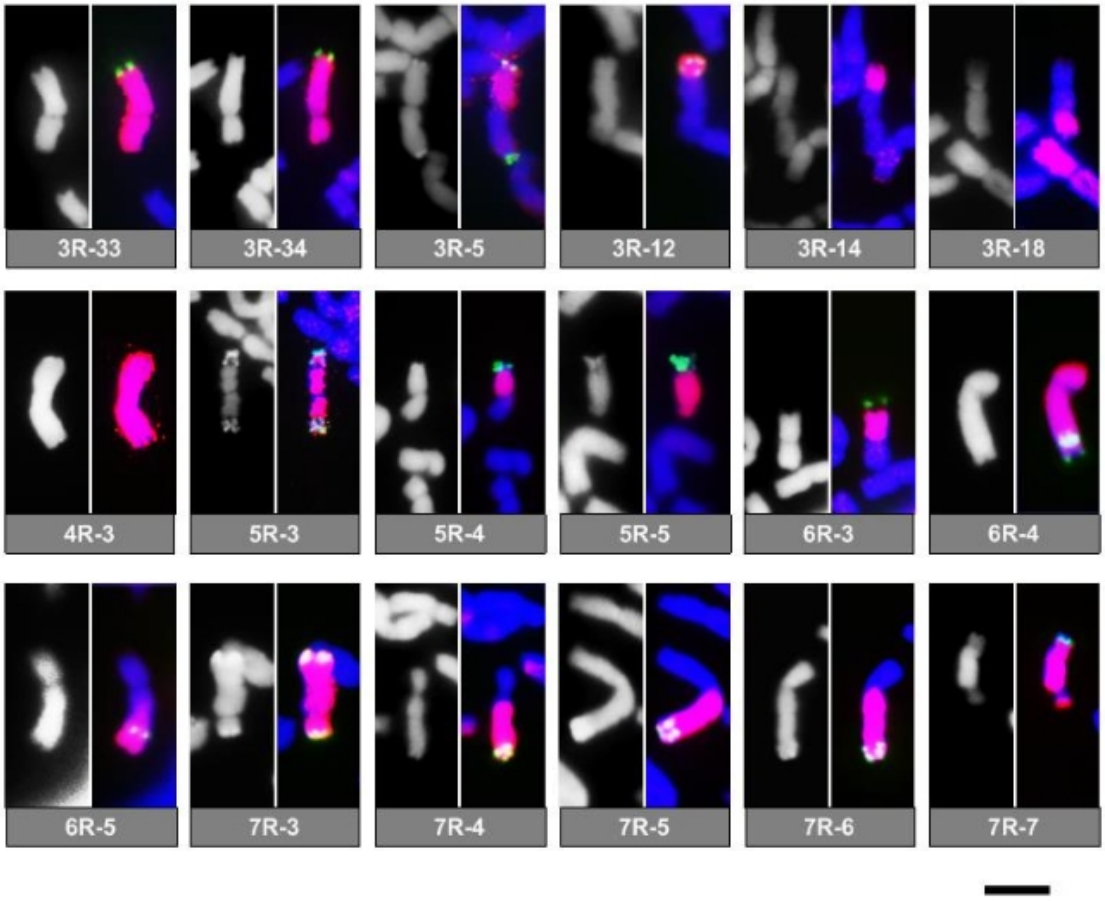

Fig. 2-4. Rye chromosome aberrations obtained during the cross-breeding of the wheat-rye addition lines carrying the gametocidal chromosome. The serial numbers in white represent the line numbers. The pink GISH signals show rye chromatin and the green FISH signals show the pSc200 repetitive sequences. Bar= 10 μm.

2R-35), 3 involved 2RL (2R-8, 2R-10 and 2R-13) and 1 involved 7RL (7R-5). Of the 14 wheat-to-rye translocations 2 had translocation points in 2RS (2R-20 and 2R-22), 3 in 2RL (2R-3, 2R-4 and 2R-28), 4 in 3RL (3R-4, 3R-6, 3R-29 and 3R-30), 1 in 5RS (5R-4), 1 in 6RS (6R-3), 1 in the 3R centromere (3R-5) and 2 in the 7R centromere (7R-4 and 7R-6). Of the 11 rye-to-wheat translocations 1 had part of the 2RS distal end (2R-5), 3 had parts of the 2RL distal ends (2R-12, 2R-23 and 2R-30), 3 had unknown 2R segments translocated onto or inserted into wheat chromosomes (2R-25, 2R-26 and 2R-27), 1 had part of the 3RS distal end (3R-12), 2 unknown 3R segments translocated onto wheat chromosomes (3R-14 and 3R-18), and 1 had part of 6RL (6R-5). The four isochromosomes were derived from 2RS (2R-14), 2RL (2R-16), 5RS (5R-3) and 7RS (7R-3), respectively.

2.4 Discussion

Except for chromosome 1R (Gyawali et al. 2009, 2010), cytological maps are still not available for the other rye chromosomes, due to the absence of dissection stocks of those rye chromosomes, i.e. common wheat stocks carrying single segments of the rye chromosomes. Although Friebe et al. (2000) produced some rye deletion stocks except for 7R, their numbers were far from sufficient for chromosome mapping and genomic studies.

The rye genome, except 1R, is highly rearranged relative to that of wheat (Naranjo and Fernández-Rueda 1991; Devos et al. 1993a; Martis et al. 2012). During the development of rye specific PLUG markers, I found that the rye genome had suffered more complex rearrangements (Li et al. 2013). To reveal exact structural changes occurred in the rye genome, I need to dissect the rye chromosomes individually.

In the BC1 progeny, I obtained critical plants with 2R and 3R for both 2C and $3C^{SAT}$, those with 4R and 6R for either of the gametocidal chromosomes, and none with 5R and 7R. For 5R and 7R, instead, I obtained semi-critical (monotelodisomic) plants. For 2R, 3R and 6R, I obtained alien double-disomic plants for a rye and gametocidal chromosomes. Eventually I expect to obtain alien double-disomic plants for the other rye chromosomes. These plants will make stable pure bred lines to which I can cross the disomic rye additions to get critical plants. After increasing the number of seeds of the critical and double-disomic lines, I will deposit those lines with NBRP (National Bioresource Project) –Wheat in order to make them available to anyone.

For all rye chromosomes 2R-7R, I found structural changes besides telosomes and isochromosomes with frequencies of 2.3%-17.4% in the progeny derived from plants disomic for a given rye chromosome and monosomic for the gametocidal chromosome. These frequencies are comparable to those reported for chromosome 1R (Endo et al. 1994) and for barley chromosomes (Schubert et al. 1998; Shi and Endo 1999). These aberrations involving rye chromosomes were surely induced by the gametocidal chromosomes.

Most of the deletions and translocations had single breakpoints but I identified two complex rearrangements, one involving two separate 2R segments translocated onto a wheat chromosome (Fig. 2-4, 2R-25), and the other involving the whole short arm and the long-arm telomeric heterochromatin of 3R between which a wheat chromosome arm was inserted (Fig. 2-4, 3R-5). Thus, by combined GISH/FISH I could find almost all rye chromosome aberrations occurred in the wheat background. Friebe et al. (2000) identified aberrant rye chromosomes induced by 2C, with a larger number of those with breakpoints in the long arm than that of those with breakpoints in the short arm. This was probably due to the fact that the small or vague terminal C-bands in the rye long arms making the detection of short arm deficiencies more difficulty than the identification of long arm deletions.

In this study, I revealed that every rye chromosome can be dissected in common wheat by the gametocidal system. I found rather many structural changes among the progeny of the mutation-inducing lines of chromosomes 2R and 3R; this is simply thanks to the larger progeny I screened. We will surely be able to obtain many more dissection lines for every rye chromosomes, and more easily by PCR analysis with rye-chromosome specific markers located in the distal regions, as conducted in the production of dissection lines for wheat chromosome 6B (Sakaguchi et al. unpublished). Such dissection lines contain chromosome-specific rye chromatins in hemizygous condition, and they will be valuable genetic stocks for the rye genetic or genomic research.

CHAPTER 3

Assignment of a mass of SSR markers to each of the rye chromosomes by using the wheat-rye addition and substitution lines

Abstract Based on the chromosome-specific survey sequences from flow-sorted chromosomes, simple sequence repeat (SSR) motifs were detected from 454 sequence reads, and the primer sets of over 26,000 SSRs were designed from contigs assembled with 454 reads (Stein et al. unpublished). To see the difference in the successful assignment of the markers to individual chromosomes between gene-associated (genic) SSRs and non-gene-associated (non-genic) ones, I conducted PCR analysis with preliminary 96 SSR primer pairs of both classes (49 from the genic and 47 from the non-genic regions) that were randomly selected from each of the sorted rye chromosomes. Using Imperial rye addition and substitution lines of common wheat, I successfully mapped 35 SSR markers to the individual rye chromosomes, 13 (26.5%) from the genic regions and 22 (46.8%) from the non-genic regions. The success rate was significantly higher for the SSR markers from the non-genic regions. Then I chose additional 2,016 primer pairs from non-genic regions (288 pairs per chromosome). By PCR analysis in the wheat-rye addition and substitution lines, I found that 822 SSR markers are specific to single rye chromosomes (75 to 1R, 125 to 2R, 124 to 3R, 135 to 4R, 130 to 5R, 105 to 6R and 128 to 7R). The success rate was 40.8% on average, varying from 35.1% for 1R and 45.5% for 4R. Of the 857 (35+822) SSR markers 187 (21.8%) had perfect SSRs with lengths of ≥20 nucleotides. Besides, 196 SSR markers seemed to be multicopy markers, showing PCR amplification for two or more different chromosomes. The most frequent motif in the 857 SSR markers was poly(GA) (27.7%), followed by poly(CA) (19.8%), poly(AT) (9.9%), and poly(CCG) (5.6%). The 857 SSR markers consisted of AT-rich and GC-rich SSRs with almost the same average repeat tracts and length and of AT/GC balanced SSRs that were slightly longer on average than the former dinucleotide SSRs. These chromosome-specific markers are surely useful in developing genetic and physical maps of rye chromosomes, and also in detecting rye chromatin introgressed into wheat.

3.1 Introduction

Rye (*Secale cereale* L.), a temperate cereal crop, is a very valuable gene pool for wheat improvement especially as a source of broad tolerance to biotic and abiotic stresses (Mohammadi et al. 2003; Bartoš et al. 2008). Despite of its agronomic importance, the genetics and genomics of rye are still under development.

In genetics and genomics, various PCR-based molecular markers are used as powerful tools for gene mapping, DNA fingerprinting, marker-assisted selection, and analysis of population structure and genetic diversity. Simple sequence repeats (SSRs), also known as microsatellites or short tandem repeats (STRs), consisted of DNA fragments containing tandem repeats of a short sequence (2–6 nucleotides), and they are a valuable source of PCR-based genetic markers (Saal and Wricke 1999). Because of their abundance, high polymorphism, high interspersion rates, codominant inheritance, enormous extent of allelic diversity, and ease of assay by PCR (Gupta et al. 1999; Kuleung et al. 2004), SSR markers have become one of the most popular PCR-based genetic markers and have been widely used in genetic and genomic studies. Since they were firstly reported in tropical trees (Condit and Hubbell 1991), the abundance of microsatellites has been found in higher plants (Gupta et al. 1996).

SSR markers are of great value thanks to their higher polymorphic information content, and therefore have been widely used in linkage mapping, diversity studies and marker-assisted plant improvement strategies. To date, SSR markers have been broadly used for genetic mapping in rice (*Oryza sativa* L.) (Temnykh et al. 2001; McCouch et al. 2002), soybean (*Glycine max* L.) (Cregan et al. 1999), maize (*Zea mays* L.) (Sharopova et al. 2002), wheat (*Triticum aestivum* L.) (Gupta et al. 2002; Holton et al. 2002; Somers et al. 2004; Song et al. 2005), and barley (*Hordeum vulgar* L.) (Liu et al. 1996; Ramsay et al. 2000; Holton et al. 2002). Compared with closely-related species, wheat and barley, few studies of DNA fingerprinting with limited numbers of SSR markers have been conducted in rye (Saal and Wricke 1999; Kuleung et al. 2004). This is mainly due to the scarcity of rye SSR markers, and this scarcity results from the limited sequence information of the rye genome.

Cereals mostly have large and complex genomes with the high content of repetitive and duplicated DNA sequences. The complexity is further augmented by allopolyploidy, i.e. the presence of two or more structurally similar chromosome sets originating from different parental species (Doležel et al. 2012). All these features

hamper the construction of physical maps, gene cloning, and *de novo* genome sequencing. Fortunately chromosome sorting technique allows identification and isolation of individual chromosomes, or fractionating the whole genome to smaller parts (Doležel et al. 2011). It plays an important role particularly in the analysis of nuclear genome structures and of specific, aberrant chromosomes (Doležel et al. 2012). Till now, the nuclear genomes of several important crops, namely rice (Lee and Arumuganathan 1999), maize (Li et al. 2001, 2004), Wheat (Wang et al. 1992; Vrána et al. 2000; Kubaláková et al. 2002; Šafář et al. 2004), barley (Lysák et al. 1999; Lee et al. 2000; Suchánková et al. 2006) and rye (Kubaláková et al. 2003), have been dissected or sorted into single chromosomes by this techniques. All the seven chromosomes of rye were sorted in euploid rye (for 1R) and in wheat-rye chromosome addition lines (for 2R-7R). Subsequently Stein et al. (unpublished) sequenced those sorted rye chromosomes individually. Then, based on the assembled contigs of the 454 sequence reads, they designed more than 26,000 SSR primer sets.

Recently comparative genetics and genomics have enabled us to conduct a series of studies on gene content and order, such as on genome zipper (Mayer et al., 2011) and colinearity or synteny (from Wikipedia, the free encyclopedia, Synteny). This is simply because of the good preservation of the gene content and order among closely related species (Devos and Gale 1997; Gale and Devos 1998b; Goff et al. 2002; Choi et al. 2004). The colinearity of common markers illustrated by comparative maps suggests the presence of a marker from one species/genus in another related species/genus (Van Deynze et al. 1998; Tikhonov et al. 1999; Kuleung et al. 2004). This so-called transferability of molecular markers has been proposed and successfully proved in many species (Peakall et al. 1998; Alvarez et al. 2001; Sourdille et al. 2001; Dirlewanger et al. 2002; Kuleung et al. 2004; Varshney et al. 2005). Therefore, primer sets designed for a DNA marker based on the sequence obtained from one species can be used to detect markers in other related species.

The high level of polymorphism of SSRs would facilitate marker development in rye. Therefore, the main objectives of this study were to test the validity of the rye SSR primer sets designed from the sequence information of the sorted chromosomes, i.e. to assign them to respective rye chromosomes by using wheat-rye addition and substitution lines; and to investigate the transferability of SSR markers from rye to wheat.

3.2 Materials and methods

3.2.1 Plant materials

I used a complete series of wheat-rye chromosome addition and substitution (for 3R) lines that have individual chromosomes (1R to 7R) of a rye cultivar Imperial (Imp) (*Secale cereale* L., 2n = 2x = 14, genome formula RR) in the genomic background of a common wheat cultivar Chinese Spring (CS) (*Triticum aestivum* L., 2n = 6x = 42, genome formula AABBDD). I obtained all these lines from Dr. Adam J. Lukaszewski, University of California, USA and from John Innes Centre, Norwich, UK. I also used euploid CS and Imp that have been maintained in the Laboratory of Plant Genetics, Graduate School of Agriculture, Kyoto University, Japan.

3.2.2 Primer design

SSR motifs were detected from the raw data of 454 reads of sorted chromosomes, and then were subject to repeat mask (RepeatMasker version open-3.1.9) analysis to eliminate redundancy. Unique SSR-containing sequences were passed to Primer3_core, a program for primer design. Primers were 18-23 nucleotides (n.t.) long (optimum size 20 n.t.), devoid of second structure or consecutive tracts of a single nucleotide, and of GC content around 45%-55% (optimum content 50%) with Tm 57-63°C (optimum Tm 60°C). The expected products range from 150bp to 400bp with optimum product size 300bp. Altogether a total of 26,539 SSR primer sets were designed. From the designed primer sets, 2112 primer pairs (49 gene-associated SSRs and 2063 non-genic SSRs) were randomly selected for PCR analysis.

3.2.3 DNA isolation and PCR analysis

Genomic DNA was extracted from fresh or frozen young leaf tissue either by the CTAB method (Saghai-Maroof et al. 1984) or by using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Purified DNA was dissolved in TE buffer, and the final concentration of the DNA was determined by NanoDrop 2000 spectrophotometer (Thermo Scientific) using TE buffer as control. The working concentration of DNA was ca. 30 ng/μL.

PCR was carried out in a volume of 12.5 μL for each well of 96-well plates with the thermal iCycler (BioRad, USA). The reaction mixture contained 25-50 ng

template DNA, 0.25 μM of each primer, 0.2 mM of each dNTP, 1× Ex Taq buffer, 1.5 mM $MgCl_2$, 3% DMSO, 1 M betaine, and 0.5 U Taq polymerase (KAPABIOSYSTEMS, USA). For all microsatellites, a touchdown protocol was used as follows: 10 s at 98°C; 7 cycles of 10 s at 98°C, 30 s at 62°C minus 1°C/cycle, 30 s at 72°C; 33 cycles of 10 s at 98°C, 30 s at 56°C, 30 s at 72°C; and 10 min at 72°C for final extension. Then for those microsatellites that were not amplified or amplified with weak bands, another PCR protocol was applied as follows: 5 min at 98°C; 40 cycles of 30 s at 98°C, 30 s at 56～64°C (optimum 60°C), 30 s at 72°C; and 10 min at 72°C for final extension.

DNA fragment sizes were analyzed by BioCal's HDA-GT12, a multiple-channel BioCal capillary electrophoresis system (eGene. Inc.), using alignment marker 15～400bp and QX DNA dilution buffer (QIAGEN) to supplement empty wells of the PCR plate.

3.3 Results

3.3.1 Comparison of the SSR markers derived from genic regions and those from non-genic regions

To see the difference in the success rate of chromosomal assignment between gene-associated and non-gene-associated SSR primers, I preliminarily conducted PCR analysis using the 49 genic primer pairs (seven for each chromosome) and 47 non-genic ones randomly selected for each chromosome (six for 6R and 7R, and seven for the rest) (Table 3-1). Using the wheat-rye addition and substitution lines, I conducted PCR analysis of those primer pairs and successfully assigned 35 of the 96 primer pairs to individual rye chromosomes, with 13 genic (26.5% success rate 26.5%) and 22 non-genic ones (46.8% success rate) (Tables 3-1 and 3-2, Fig. 3-1). The success rate was significantly higher for the non-genic primer pairs than that for non-genic ones (chi-squared test, $p=0.0016<0.05$). Nine primer pairs showed PCR amplification in two or more different wheat-rye addition and substitution lines, i.e. multiple-chromosome-specific markers (Table3-1, Fig. 3-2). Of those nine markers,

seven were from non-genic regions and two from genic regions, which further proved the superiority of non-genic markers in developing rye-specific markers.

Table 3-1. Preliminary chosen 96 rye SSR primer pairs and their assignment to individual rye chromosomes

Running no.	ID	Sorted from*	Forward primer (5'-3')	Reverse primer (5'-3')	Genic or non-genic	Repeat sequence	PCR-product (bp)	PCR amplification in		Assigned chromosome
								wheat-rye lines	wheat	
1	GPVJ0NR01DUQ2Z	1R	GAACAAAAGGATTGGGGCAT	GCATGCAATGTTGGAATCGT	non-genic	(TTC)6	150	+	-	1R
2	GP3EJ2G01CV5TE	1R	ACAGGGGAAGGGAGATGAGT	GAGTTTGTTGCTTGGTTGGG	non-genic	(TA)6	152	+	+	1R
3	GPVJ0NR02IHECX	1R	CCAGCGAAGCTTTTGCTACA	AAAGCTCCCAATCAGACACTG	non-genic	(ACG)6	150	+	-	1R,2R,3R,4R,5R,6R.7R
4	GP3EJ2G01B8H2Q	1R	AGCTTAACACCACCAAGAGC	TCTGGCTGCTGTTCTAAGGAA	non-genic	(GA)6	153	+	-	1R,5R
5	GP3EJ2G01AXL3S	1R	TCGAGAGGAATGGGTAGCAC	TGCTAGGCGGAGCTTCTTTA	non-genic	(GA)10	151	+	+	1R
6	GPVJ0NR02FHXA6	1R	TCTGGTTTCTACTACATGGCTG	GGAATGAATTAGGGCGATCA	non-genic	(TG)11	157	-	-	
7	GPVJ0NR01EN37A	1R	TGGTGATGTCCATAGGATGC	CTTCTCTCTTGAAAGGACGCA	non-genic	(AGG)5ggct(AGG)5	151	-	-	
8	GPVJ0NR01BIPCN	1R	TGTGCATTTAACCACAAGGG	AATCCTGTCCGACTGTGGTG	genic	(AAT)6	153	+	+	
9	GPVJ0NR01DIMQX	1R	CCATCTGTACTCCCACCTCA	TGTGCTGATACTGTTGTTGACG	genic	(AG)6	199	-	-	
10	GP3EJ2G02F4047	1R	CAATCAACAGCACATCAACC	AGGCAGAGCTCAAGCGTTAT	genic	(AG)8	165	-	-	
11	GPVJ0NR01CJU75	1R	TCGAACAGTAACACCTCCCC	ACGACAGACCAGCCAGTGAT	genic	(CCG)5	199	+	+	
12	GP3EJ2G01BER65	1R	TCTTTTTGTTTGCAGGAGGC	CTCCCCAAGAATGTCAAGGA	genic	(AGC)5	227	-	-	
13	GP3EJ2G02HXNAL	1R	AGGAAAATGGTGACGGTACG	GGACTTCCTTGGTGCTTCAG	genic	(CA)6	165	+	+	
14	GP3EJ2G02I3DGV	1R	TGACGATGATGGTTACCTCG	CGTGCAGCTGCGTGTATTAT	genic	(CA)8	216	+	-	1R
15	G5EUT7Q02GQ42P	2R	CACACAACAGACGCTACAGAG	GATCCGGTGAGAGAGAGCAT	non-genic	(CT)6	152	+	+	
16	G5EUT7Q01D1ITX	2R	CATTATAATCAGGTCCCACCAG	AGAACTCCGAGGAGAACATTG	non-genic	(ATAG)5	153	+	-	2R
17	G5EUT7Q01BEEAO	2R	CCCTAGTTACGAGTTGATGGTCA	AATGTCAAGCATCGACTTGC	non-genic	(AC)6cctagttgcgggtcgatgggcaatttggcaactggggctgtcagcaa(AC)8	152	+	+	

18	G5IHSOS01EGMYZ	2R	TGTCTCCACGAAGGGAGTCT	GGTGAGATGTATGCCTCTCTGG	non-genic	(C)13an(AC)6※	150	+	-	2R
19	G8EP86W01C0JX5	2R	CCTACACAAGGTTGACCTCATT	TATTCATGAAGGTGGTGGCA	non-genic	(TA)8	151	+	+	
20	G5EUT7Q01B0JDE	2R	GGGGAGGAAGATGATATGGA	TCGATGTCGCCTCAAGTACC	non-genic	(AG)9	150	-	-	
21	G5EUT7Q02IBG1C	2R	AGGGTCTCCTGCTTTGAACA	GGAATCAATCATCTCCCAGC	non-genic	(CTC)7	167	+	-	2R
22	G4YLJF201EC2XK	2R	CTAGTCGATGAGGCCAAAGG	GCCTACCCCGTACAAGGAAA	genic	(CGT)5	165	-	-	
23	G4YLJF202FPN17	2R	TGCCTCGGGAGGTAATACAA	TCGGACAAGCCATTTCAGAG	genic	(AG)6	153	+	+	
24	G5EUT7Q01APPAP	2R	AATGTAAGCTGCCTGATGGG	CCATGCCTCTTACACCTTCC	genic	(TC)7	179	+	-	2R
25	G5IHSOS01CCANU	2R	AGTTGGAGGTTTTTGAGGCA	GGCCGACTTTGTAGTTCTGG	genic	(GA)6	201	+	+	
26	G5IHSOS01AGNC8	2R	ATGGTGTACTGAGCCTGCTG	ATGTGCAGTTCGCCATGAT	genic	(TGT)5	151	+	+	
27	G5EUT7Q01CI8BA	2R	AGAAGAAAACCGGCCTTGTC	GGGCTGGAAAAGAACGTACA	genic	(TC)7	182	+	+	
28	G4YLJF201BGU05	2R	TGGGAGCTTACGAATTGCAC	AGCGTGCCTCCTCTCATCTA	genic	(GA)6	231	+	+	
29	G40IFLJ02G0RAC	3R	TGCGCGTGAGAAGTCTACAA	GAAGGTGAGAACTCGATCTTGA	non-genic	(GT)8	156	+	+	
30	G49NJZT01DPNJ6	3R	GCACAACAAACGGCATATTG	GGTGCCGAAGATGTGCTATT	non-genic	(CG)7(CA)8	151	+	+	3R
31	G40IFLJ02JXE63	3R	AGGATCGTGGGTGGGATATT	ATATGAGGAGCGGGAGTACG	non-genic	(TA)7	153	+	-	3R
32	G5IHSOS02JEXKD	3R	AATGCGTCACTGGCATAGC	ATCTCGCCAGAGTTTCGTTG	non-genic	(AG)6	151	-	-	
33	G40IFLJ02I9Y74	3R	CTACCTTGCTCATGTCGTCCT	TCATCTACATCCTCCCCTCG	non-genic	(TG)11	156	-	-	
34	G5IHSOS02H1HPO	3R	TCTGACACCACCTGCACTCT	CACGATGGCCAGCAAAAA	non-genic	(CT)6	155	+	-	1R,3R,6R
35	G49NJZT01CI6MK	3R	TTCCATTTGGATCGGGAAC	TCAGGCCAAATCGGAAGTC	non-genic	(CAGC)5	152	+	-	3R
36	G5IHSOS02H0TB5	3R	AAGGAAGAGATCATGGGCAG	ATGCGCAGGTTTCAACTCAT	genic	(GAG)7	192	+	-	3R
37	G5IHSOS02GO5W3	3R	GAAAAGCTTCAGCATGGACG	ATGAGTCCGTCGGAGAGGTA	genic	(GTC)5	182	+	-	1R,3R,5R,6R,7R
38	G40IFLJ02HVCVN	3R	GGCTTCTGCCGAGTTAACAG	GCTTTAAGATTCGCGACCAG	genic	(GT)7	228	+	+	
39	G5IHSOS02IQ4MD	3R	ACTTTGAAAAGCGGGTAGGC	CTTGAGGATGTTGCTGACGC	genic	(AGC)5	222	+	+	1R,2R,3R,4R,5R
40	G5IHSOS02FYU1E	3R	GCATTCCTGGCACTTGAAC	AATCCTAAGCTTGGAAGGGC	genic	(CGG)5	188	+	+	
41	G49NJZT02JUWRX	3R	AAGCAGAAGAAAAGCAAGCG	ATAGGGGACGGGGTTACTGA	genic	(AAG)7	231	+	+	

42	G5IHSOS02IZ6S4	3R	TGGTGGAGAAAGATGGAAGC	GGAGCTTATGATTGATCGCC	genic	(CA)6	194	+	-	3R
43	G562D2H01DXKO7	4R	CGATAAGCAAGCTGAGTTACGG	ATACCCTCGGTCTTGGTTTCC	non-genic	(AG)8	150	+	+	4R
44	G562D2H02F953F	4R	ACAGATAAACATGGCAGGGG	TGATGTTGTGTCTTCTTAGGCG	non-genic	(AT)6	150	+	-	4R,6R,7R
45	G613Y7R01BJWJ3	4R	AACAAATGGGCAAGCACGTA	AGGGCATTAGGAGGAGGGAT	non-genic	(TG)13	150	+	-	4R
46	G613Y7R01EOMI6	4R	ACTTGGGAGATGGTGAAGCG	GGCTGAACGTTTCTTCTTCC	non-genic	(AG)6	154	+	-	1R,3R,4R
47	G69HL7G02GOY1L	4R	AACCTAGGGAAAAGGTGGTAGC	GGTCTGCAGTCAAAGACTATGTG	non-genic	(TA)7	150	+	-	4R
48	G5Y8MOU01DTV9J	4R	AAGCCCTTCAATGAGCACAA	GGTTTCATTAGACCATTCCG	non-genic	(TA)6	150	-	-	
49	G69HL7G02JGJM7	4R	AGGGCCAATTTTAAGAGAGAGG	TGTTTTTCCTAGTGCTAAGGCG	non-genic	(CT)8	150	+	+	
50	G562D2H01BI7ZJ	4R	ATCAAGAACCATGAGCACCC	CCTGATCCTCCATGATACCC	genic	(AGG)5	160	+	+	
51	G5Y8MOU01DB3C3	4R	AAGCATACCCTGCGAGAAGA	TCCTCAAGTCCAGTGCTCCT	genic	(GTC)9	182	+	+	4R
52	G562D2H01C6B2T	4R	ATGGAGCCCAGCAACTAAGA	GGTTTCTGGTCTTTTGTCTGTG	genic	(TA)7	201	+	+	
53	G69HL7G02JFPHS	4R	CCTGCACCACATGTAACTGG	CGAAGAATTGGACATGAGTGC	genic	(CAG)5	289	+	+	
54	G5Y8MOU01A5K2O	4R	ACTGGGGAGATGATCGTGAC	ACGTGAAAGCAACTCCGACT	genic	(AAG)5	214	+	+	
55	G69HL7G02F4ECN	4R	CGCGTCCATTTTTACCCTTA	ATTTTGTGAGCTGTCCGACG	genic	(CT)6	284	+	-	4R
56	G69HL7G02FUAZ4	4R	CAGACCAATCTGCTGCTTCC	TCACCCTGAGGCTGTTCTTC	genic	(CCG)5	217	+	+	
57	G6Z8DEW02HCMO6	5R	AAAAACCAGCCGTCGAAC	GAATTCCGGCGTGATACTGT	non-genic	(CT)6	179	+	-	5R
58	G6Z8DEW01CTA7T	5R	AAAAACCTATGCCCACATCG	GGATTAGGCTACCCGTGACA	non-genic	(CCG)5	348	+	-	5R,7R
59	G6Z8DEW01BABDL	5R	AAAAAGCCATAAGCTCGCCT	GGCGACAGATTGTAGTGGGT	non-genic	(GCC)8	156	+	-	5R
60	G6Z8DEW02FV92Q	5R	AAAAATCGCCTATTCACCCC	AACTGCTCGAACAAAAGCGT	non-genic	(GCT)5	201	+	+	
61	G6Z8DEW01DQAMW	5R	AAAACAAACACTGTTCCCCG	GGAGAAGCGAAGGGGATATG	non-genic	(CGC)6	177	+	-	5R
62	G6Z8DEW02HK6YZ	5R	AAAACACCTTCTTCGCGGTA	TAGGGTTGAAATTCGGCAAC	non-genic	(CTT)5	273	-	-	
63	G613Y7R02FROPY	5R	AAAACATACACTCGCCTCGC	CTACATGGCTCGCTTGTCAG	non-genic	(AAT)5	166	+	+	1R,3R,6R
64	G5Y8MOU02G2DX1	5R	AAAAGGTGGCTGGCTGTCT	TTATCTCTGGGTTTGGCAGG	genic	(CGC)5	380	-	-	
65	G5Y8MOU02JDZDO	5R	AACCAAGGGTACCAGTGTGC	ATGTCTGCCTTGATTTTGGC	genic	(ACCA)5	254	+	+	
66	G75F86X02F7OLX	5R	AACCAATCAGAACTCCACGG	CGTCTTTTCTCTCTCCCACG	genic	(CGG)5	272	+	+	
67	G6Z8DEW01AR9AI	5R	AAGAAGGTCATCATCACCGC	GCACCACAGGACAGTCAGAA	genic	(TA)7	330	+	+	

68	G613Y7R02IFCC5	5R	AAGAGAGAAGGAGGTGAGAGATG	GGATCTTCTGCATCTGGAGC	genic	(GGC)6	273	+	+	
69	G6Z8DEW01EQRRD	5R	AAGAGTTGATGATGACGGGG	CCCTTCTTTTTGCTGCAGAT	genic	(GCT)5	205	+	-	5R
70	G75F86X02IDF0N	5R	AAGCAAAGAGAGAGAGGGGG	GGGCTCCATGAGCCTAATTT	genic	(GA)6	169	+	+	
71	G7FJU1X02I3XTI	6R	AAAAACAACGACGACCAAGG	AAGTGCAGGAGGAGAACGAA	non-genic	(GGTT)5	212	+	-	6R
72	G7DL51X01CV1Z1	6R	AAAAACAGGGGAGGGGCAT	ACCTGCCACGTTCTTCAGTT	non-genic	(TC)9	156	+	+	6R
73	G8EP86W02I73UN	6R	AAAAACCAGAAGCGCACACT	CACGTGGAAACGATGTCTTG	non-genic	(TTA)7	278	-	-	
74	G7DL51X01BZDW8	6R	AAAAACGGTCGTGTCCTACG	TTAAAACGACCAAACCGAGC	non-genic	(ATT)5	196	+	+	6R
75	G7DL51X02HWHVX	6R	AAAAAGCAGAGTAGAGCAGGC	TTTCGGATTTCCATCGTCAC	non-genic	(GGC)5gtgcggcaagcaggggcggcggcgtgcagagcaggagcaggcaggggc(G)13	327	+	+	
76	G7DL51X02I5XG4	6R	AAAAAGGACCAGGATACCGC	GATGCATTTGGACGAGCAC	non-genic	(CT)6	165	+	+	
77	G8EP86W02I6PCT	6R	AAACTTCACCAACGTGGACC	TGCTGCCTCTTGTTTGAATG	genic	(TG)6	269	-	-	
78	G8EP86W02IQCEY	6R	AAAGCGAGGTAGCCGTGAAT	GCCCTTTATGTGATGTCGCT	genic	(GCA)5	390	+	+	
79	G7FJU1X01BPIOM	6R	AACAACTTCCCCTCCAGGAC	TGTGGACAGTATGCTCCCAA	genic	(CCTCA)5	231	+	-	6R
80	G7DL51X02FOZFC	6R	AACGACAGCCGATATCCAAC	TCATCCTACATTTCGGGAGC	genic	(GT)6	317	+	-	6R
81	G7DL51X02F4D64	6R	AACTCCGAGGCACACTTCAC	GCTTCATCTCGACGACGGTA	genic	(GCG)5	187	+	-	6R
82	G7FJU1X01ASC1K	6R	AACTTCCTCCACATCAAGGG	AAGGCCCATGGACTCTCTTT	genic	(GAG)5	161	+	+	
83	G8EP86W02FKM1S	6R	AACTTCGCATTCTGAACAGC	TTGTCTTTCCCAACCACCTC	genic	(ATT)5	248	+	+	6R
84	G76ZXPN02FX8X2	7R	AAAAAGCTGGTAGTGGGGGT	CTCCAATTCCCGTAGTGAGTC	non-genic	(CAG)5	153	+	+	
85	G73J0XM01EOZS2	7R	AAAAATCCTAATCCGGACGC	CGGACCATCTCCTTCTCGTA	non-genic	(CT)6	151	+	-	7R
86	G73J0XM01EUWJL	7R	AAAAATGAGCCACTTCTGCC	ATCAGCCACTTCTGCTCGAT	non-genic	(CT)6	169	+	-	7R
87	G73J0XM02HX3BS	7R	AAAACCCTCCGCAGAAGTG	GGTCAAGAGCTTCGATTTGC	non-genic	(CGC)5	269	+	+	7R
88	G73J0XM02GPRXO	7R	AAAACGGCGAACCACATAGT	TAACCATGCACCCAGTGAGA	non-genic	(CA)6	260	+	-	7R
89	G76ZXPN01CSAQG	7R	AAAACTGGCTCCACCCTTCT	TGTTTGCATACCACGCAGAT	non-genic	(TC)9	233	+	+	

90	G76ZXPN01BDUWI	7R	AAACTGTGAGGGGCAGAGTG	CCAAACAAGCTTGGCTTCTC	genic	(AT)11	316	+	-	7R
91	G76ZXPN02G9YDJ	7R	AAAGTGACGTGCAACAGCAG	CCGACTCCTTCTTCGTCAAC	genic	(GTGTAG)5	183	+	+	
92	G76ZXPN02FKLDF	7R	AACAATTGGCAGCAAGGAAC	TCTCTCCGAGAATCGCCTGT	genic	(TC)7	297	+	-	7R
93	G73J0XM01BIJYS	7R	AACACAGCTCCCACCTCAAC	AGTCAACTGTCAATGGTGGC	genic	(ACAG)5	357	+	+	
94	G76ZXPN01BQQXK	7R	AACAGGAGCGGGACTACATC	GCACCTGCCCAAGTAAAAGA	genic	(TTTA)5	336	-	-	
95	G73J0XM01ANWFY	7R	AACGCCACCTTGTCAGTTGT	AAATAAAAGAACCCACCCCG	genic	(TA)9	307	+	+	
96	G73J0XM02IGP64	7R	AACTACTGGAGGTACGCCGA	CGCAAGAACAAAGAACGACA	genic	(GCA)6	336	+	+	

* Sorted rye chromosomes from which the SSRs were found.

※ The “n” in the motif means ambiguous at this nucleotide site.

Table 3-2. PCR analysis of the 96 SSR primer pairs using the wheat-rye addition and substitution lines

Category		No. SSR primer pairs from sorted rye chromosomes							
		1R	2R.	3R	4R	5R	6R	7R	total
Genic	No. Tested	7	7	7	7	7	7	7	49
	No. successful*	1	1	2	2	1	4	2	13
Non-genic	No. Tested	7	7	7	7	7	6	6	47
	No. successful*	3	3	3	3	3	3	4	22

* Numbers of SSR markers successfully assigned to single rye chromosomes.

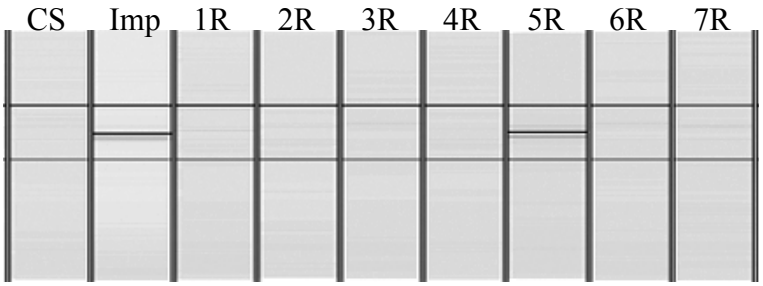

Fig. 3-1. An example of PCR analysis to assign a rye-specific SSR marker (RSSR_59) to a critical rye chromosome (5R). CS: Chinese Spring wheat, Imp: Imperial rye, 1R-7R: wheat-rye chromosome addition and substitution (for 3R) lines.

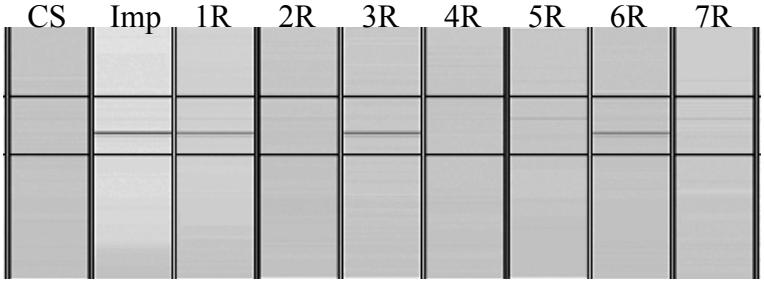

Fig. 3-2. An example of PCR analysis to assign a rye-specific marker (RSSR_34) to multiple rye chromosomes (1R, 3R and 6R). CS: Chinese Spring wheat, Imp: Imperial rye, 1R-7R: wheat-rye chromosome addition and substitution (for 3R) lines.

3.3.2 Assignment of a mass of rye-specific SSR markers to the individual rye chromosomes

Based on the above finding, I chose additional 2,016 SSR primer pairs from non-genic regions, 288 each for the rye chromosomes (Table S3-1). By PCR analysis using the wheat-rye addition and substitution lines, I found a total of 822 primer pairs

that were successfully assigned to single rye chromosomes: 75 to 1R, 125 to 2R, 124 to 3R, 135 to 4R, 130 to 5R, 105 to 6R, and 128 to 7R (Tables S3-2 and 3-3). The primer pairs of these chromosome-specific markers were mostly designed based on the sequences from the corresponding sorted chromosomes and vice versa. However some of them were designed on the sequences from the non-corresponding sorted chromosomes. For example, 74 of the 75 markers assigned to 1R were from the sorted 1R chromosome, but one marker was from the sorted 2R chromosome; conversely, 101 (35.1%) of the 288 markers from the sorted 1R chromosome were successfully assigned to single chromosomes, and 74 of the 101 markers were specific to 1R and the rest were specific to 2R to 7R (Tables S3-2 and 3-3). The success rates of chromosomal assignment varied from 33.7% for the 288 primer pairs from the sorted 6R to 45.5% for those from the sorted 4R, 40.8% on average, and the percentages of the chromosome-specific markers derived from the corresponding sorted rye chromosomes were 73.3% (1R) to 100% (6R).

Table 3-3. Summary of chromosomal assignment of the 822 SSR markers

Source	No. SSRs tested	No. SSR markers assigned to a rye chromosome								Success rate (%)*	Specificity (%)**
		1R	2R	3R	4R	5R	6R	7R	Total		
Sorted 1R	288	74	5	2	5	1	7	7	101	35.1	73.3
Sorted 2R	288	1	120	0	0	0	0	0	121	42.0	99.2
Sorted 3R	288	0	0	119	1	0	0	0	120	41.7	99.2
Sorted 4R	288	0	0	1	129	0	1	0	131	45.5	98.5
Sorted 5R	288	0	0	1	0	129	0	0	130	45.1	99.2
Sorted 6R	288	0	0	0	0	0	97	0	97	33.7	100.0
Sorted 7R	288	0	0	1	0	0	0	121	122	42.4	99.2
total or average	2016	75	125	124	135	130	105	128	822	40.8	96.0

* Total no. SSR primer pairs assigned to a rye chromosome) / (Total no. SSR primer pairs tested).

** No. SSR primer pairs assigned to a corresponding rye chromosome) / (Total no. SSR primer pairs assigned).

Meanwhile, 187 primer pairs were amplified in multiple wheat-rye addition and substitution lines (Fig. 3-2): 65 were amplified in two different lines, 31 in three lines, 16 in four lines, 13 in five lines, 12 in six lines, 50 in all seven lines (Tables S3-3 and 3-4). On the other hand, I could not assign 73 rye-specific markers to any of the rye

chromosomes due to inexplicable PCR amplification results, i.e. amplification in none of the wheat-rye addition and substitution lines despite amplification in euploid rye (Fig. 3-3).

Table 3-4. Summary of chromosomal assignment of the 187 SSR markers assigned to multiple rye chromosomes

No. multiple chromosomes to which a SSR assigned	No. SSRs assigned	No. SSR markers derived from						
		sorted 1R	sorted 2R	sorted 3R	sorted 4R	sorted 5R	sorted 6R	sorted 7R
2	65	9	8	15	6	11	6	10
3	31	2	6	4	3	5	5	6
4	16	1	4	3	1	4	1	2
5	13	1	0	3	1	3	2	3
6	12	0	0	1	3	2	2	4
7	50	1	5	4	9	4	8	19
Total	187	14	23	30	23	29	24	44

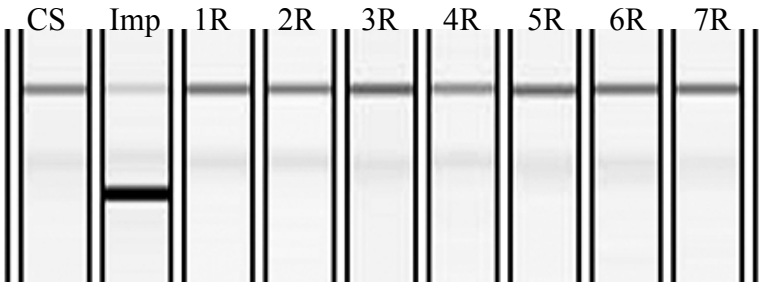

Fig. 3-3. An example of PCR analysis showing an inexplicable result of rye-specific SSR marker (RSSR-Chr_1_7). CS: Chinese Spring wheat, Imp: Imperial rye, 1R-7R: wheat-rye chromosome addition and substitution (for 3R) lines. Note there was no PCR amplification in any of the wheat-rye lines.

3.3.3 Frequency and length variation of rye-specific SSR markers

Figure 4 shows the relative frequency of the 12 most abundant motifs among the 857 newly developed rye-specific markers. The most frequent motif was poly(GA)

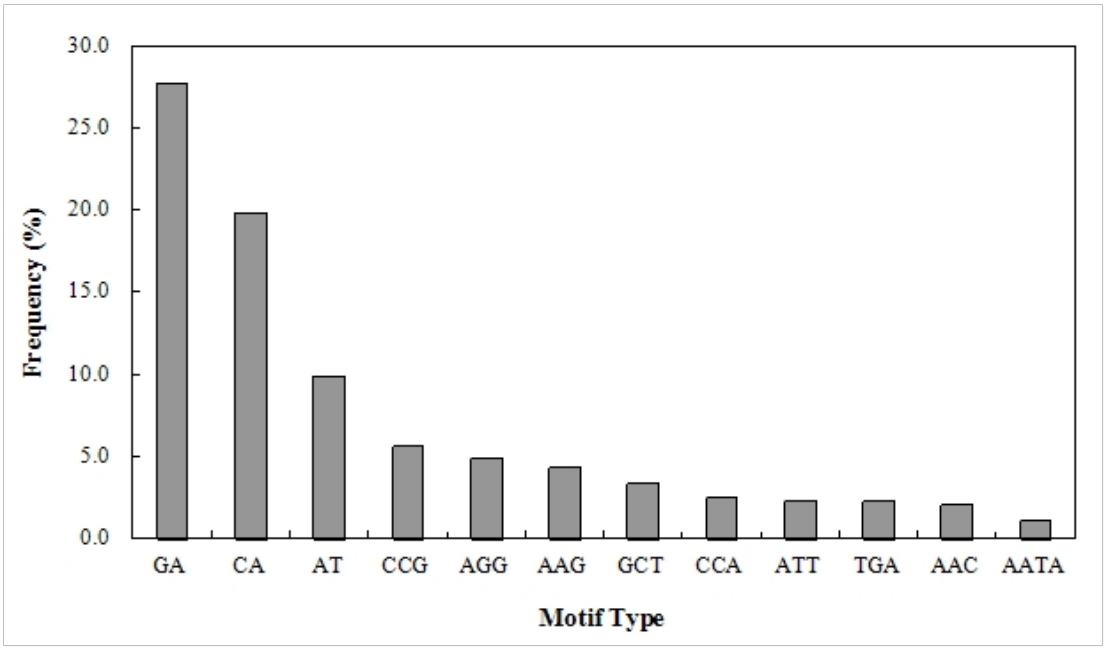

Fig. 3-4. Frequency (%) of the 12 most abundant SSR motifs in the 857 rye-chromosome-specific SSR markers.

(27.7%), followed by poly(CA) (19.8%) and poly(AT) (9.9%). The proportion of tri-nucleotide motifs constituted about 29% of the new markers, with the top three motifs, ploy (CCG) (5.6%), poly(AGG) (4.9%) and poly(AAG) (4.3%). In addition, some markers had poly tetra-nucleotide motifs, from poly(AATA) (1.1%) down. All di- and tri-nucleotide motifs, and most of the tetra-nucleotide motifs were represented among the new markers, with the exception of poly(GGGC), poly(AATT), poly(CCAG), poly(ATGC) and poly(AAGT).

Among the three categories of SSRs, namely AT-rich (greater than 50% A or T in the motif), AT/GC-balanced and GC-rich motifs, the longest average repeat (7.5 repeat units, 15.3-nt length) was found in the AT/GC balanced SSRs. The AT-rich and GC-rich classes had almost the same average repeat numbers (～6) and average lengths (～17 nt) (Fig. 5). The longest uninterrupted repeat was a di-nucleotide $(AC)_{24}$ (GP3EJ2G01CLG10 or RSSR_Chr1_117). Among the 857 new SSR markers, 187 (21.8%) were perfect repeats (≥20 nucleotides).

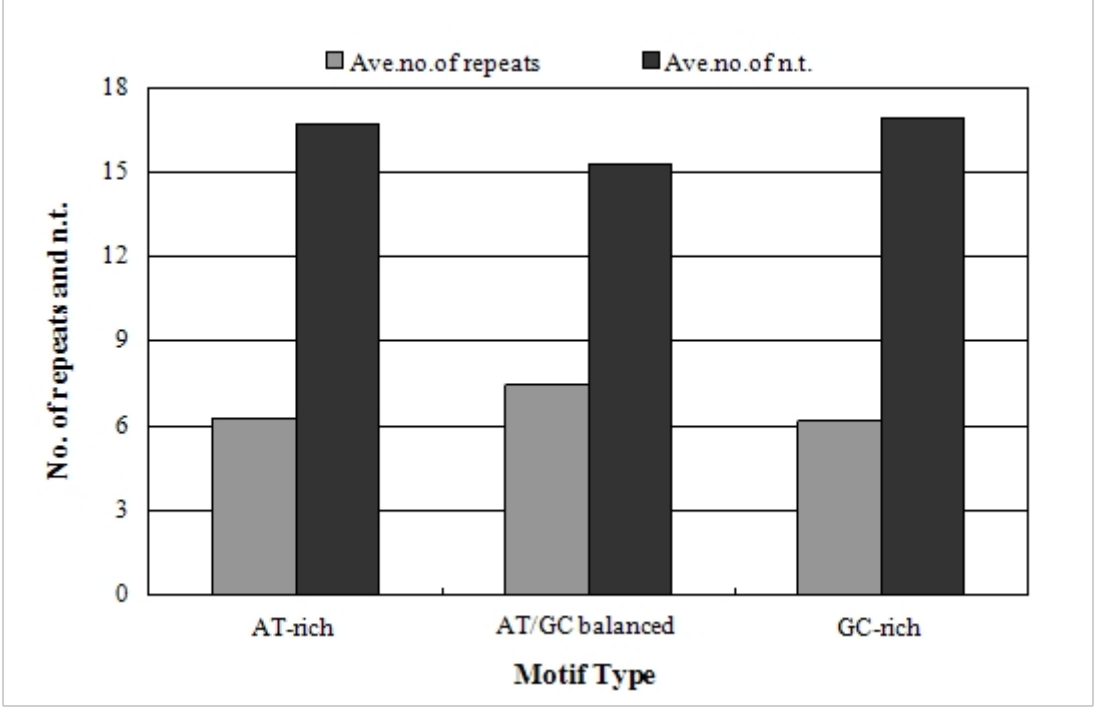

Fig. 3-5. Relationship between the motif type and the length of repeat tract in the 857 rye-chromosome-specific SSR markers. The AT-rich (> 50% A or T) group accounts for 26.8% of the markers, the AT/GC-balanced (only di- and tetra-nucleotide) group for 49.9% of markers, and the GC-rich (> 50% G or C) group for 23.3% of the markers.

3.4 Discussion

In the present study, SSR primers in two different categories, namely those from genic regions and those from non-genic regions, were used for the development of rye-specific markers. The results demonstrated that it is relatively inefficient in developing rye-specific markers to use SSR primers designed in the genic regions. Since all the sequence information on the gene-associated SSRs are from the rye "genome zipper", these SSRs represent the genic regions that have syntenic relationships among the genomes of reference species, such as brachypodium, rice and sorghum; therefore those SSRs are not specific only to rye, i.e. those gene-associated SSR regions are highly conserved between rye and wheat, and thereby not suitable for developing rye-specific markers.

Chromosome-specific markers are useful for chromosome mapping. In this study, 857 rye-chromosome specific markers were developed from the 2112 rye SSR primer

pairs. This success rate (40.8%) is much higher than those reported in other studies attempting to acquire rye-specific markers, e.g. directly from wheat and rye EST primers (Xu et al. 2012), excepting the success rate of 55% attained by using the PLUG system (Li et al. 2013). Therefore it is a reasonable to use SSRs in developing species- and chromosome-specific PCR-based markers. Besides, it was proved in this study that flow sorting, so-called flow cytogenomics (FC) is a very effective approach to dissect or isolate nuclear genomes into single chromosomes with a high purity (Doležel et al. 2007; Doležel et al. 2012; Grosso et al. 2012). Most (73.3-100%) of the SSR markers obtained from the sequences of sorted chromosomes were assigned to the corresponding rye chromosomes (Table 3-3). The lowest rate for 1R is probably due to the fact that the 1R chromosome was flow-sorted from euploid rye itself, in which chromosome complement 1R is not strikingly different in size from the other chromosomes and therefore the sorted 1R fraction might have been contaminated with the other rye chromosomes. Whereas the other rye chromosomes were isolated from the wheat-rye addition lines, the rye chromosomes are significantly larger than the wheat chromosomes and therefore the individual rye chromosomes might have been flow-sorted in high purity.

The fact that 196 rye-specific SSR markers showed PCR amplification in two or more different rye addition and substitution lines suggests the presence of multiple-copy SSR markers on different chromosomes. Such SSR markers were also reported in rice (McCouch et al. 2002), and some of the barley ESTs were mapped to multiple chromosomes (Nasuda et al. 2005).

Although most of the rye-specific SSR markers were successfully mapped to individual rye chromosomes, 73 of such markers could not be mapped to any of the rye chromosomes by using the wheat-rye addition and substitution lines. In other words, the 73 markers showed PCR amplifications in Imperial rye but did not show the corresponding PCR amplifications in any of the wheat-rye addition and substitution lines. These unexpected PCR amplification results might be attributable to genetic polymorphism between the line of Imperial rye used as the control in this study and the one used for the development of the wheat-rye addition and substitution

lines. Alkhimova et al. (1999) and Li et al. (2013) had the similar reports in their previous studies.

The investigation on the distribution and variability of microsatellite sequences in the genome of a given species can elucidate the genetic history of the species from the standpoint of evolution and artificial selection (Cho et al., 2000). The structure and length of SSRs are considered to be the key factors affecting microsatellite variability (McMuraay 1995; Brinkmann et al. 1998; Cho et al. 2000). Many comparative evolutionary studies so far focused on marker polymorphism emphasized the differences in the genetic variability of microsatellite sequences with different motifs and sources (random genomic clones versus expressed sequence tags). It was shown in the early studies of human microsatellites (Weber 1990) and has been confirmed in several different organisms that the variability of SSR markers correlates well with the length of the tandem arrays (Goldstein and Clark 1995; Innan et al. 1997; Cho et al. 2000), i.e. simple sequence length polymorphism (SSLP), which can be used to understand the genetic variance between two individuals in a certain species (King and Motulsky 2002; Rosenberg et al. 2002). In the present study, I found some markers that showed different PCR amplification in band size between the control Imperial and the wheat-rye addition or substitution lines (data not showed). Most of such markers had either the large numbers of repeat units or long nucleotides motifs. Besides, I found poly(GA) motifs (27.7%) was the most abundant motifs among the 857 newly developed markers, and poly(CCG) motifs (5.6%) was the most abundant tri-nucleotide motifs. These results were completely consistent with those in rice (McCouch et al. 2002). While markers with the poly(AT) motif (9.9%) amplified poorly in general, and a similar tendency was reported by Temnykh et al. (2001) in rice, although the poly(AT) blocks are the most abundant and variable microsatellite sequences. In addition, AT-rich and GC-rich motifs had the longest repeat motifs with almost the same average numbers of repeat units and the same average lengths of the repeated sequence. In rice, however, different studies reported different proportions of AT-rich or GC-rich motifs among the developed SSR markers (Cho et al. 2000; Temnykn et al. 2001; Morgante et al. 2002; McCouch et al. 2002). The categories of

SSR motifs seem to be very different in proportion between SSR populations originated from different sources. In this study I obtained 187 (21.8%) long microsatellites (≥20 nucleotides) that contained perfect SSRs. These markers would become valuable tools for genetic polymorphism studies in rye because the longer perfect repeats are highly polymorphic (Temnykh et al. 2001).

Over the past two decades, it has been demonstrated that SSR markers show a certain degree of transferability among related species. In general, high levels of marker transferability was detected from EST-derived SSRs (Thiel et al. 2003; Cordeiro et al. 2001; Gupta et al. 2003), which reflected the conserved nature of coding sequences compared to non-coding genomic DNA (Varshney et al. 2005). However, the marker transferability was not high for the SSRs detected directly from genomic source (Röder et al. 1995; Peakall et al. 1998; Sourdille et al. 2001; Kuleung et al. 2004). In the preliminary PCR analysis of the present study, I found that more than 1600 out of 2112 rye SSR primer pairs were amplified in wheat with a high success rate (76%) (Tables 3-1, S3-2 and S3-3). This implies that the transferability of SSR markers from rye to wheat is high, although further confirmation is needed.

In summary, the abundant sequence information from sorted rye chromosomes provided a super opportunity for the systematic development of molecular markers including SSR markers. In this study 857 out of the 2112 primer sets of SSRs were successfully mapped to single rye chromosomes with a high success rate of 40.8%. These rye-chromosome specific markers would be very useful not only in genome mapping, gene tagging, genome analysis and the detection of chromosomal segments and genes, but also in the estimation of genetic relatedness and comparative mapping among Triticeae species.

GENERAL DISCUSSION

In cytogenetic studies, chromosome karyotyping is a major part of the research including chromosome structure, chromosome identification and chromosome nomenclature. The DNA content (1C) of the rye genome is 9.5 pg (Bennett and Smith 1976), approximately 33% larger than that (ca. 6 pg) of the diploid wheat genome (Arumuganathan and Earle 1991). Besides, massive blocks of terminal heterochromatin are present on each of the rye chromosomes (Lima-de-Faria 1952), so the rye chromosomes are distinguished easily from wheat chromosomes in wheat × rye hybrids (Gill and Friebe 2009). The individual rye chromosomes were cytogenetically identified by C-banding analysis of wheat-rye (cv. Imperial) addition lines (Gill and Kimber 1974) and by heterochromatic banding patterns using Leishman staining (Darvey and Gustafson 1975). Mukai et al. (1992) showed a detailed characterization of a rye C-banded karyotype in Chinese Spring wheat-Imperial rye addition lines. In addition, homoeologous pairing involving wheat and rye chromosomes was detected by C-banding and GISH in wheat-rye derivatives (Fernández-Calvín et al. 1995). Besides C-banding patterns, Schlegel and Gill (1984) introduced N-banding procedure to rye chromosomes and found three chromosomes additionally marked, including 2R, 3R and 6R (see detailed description in Schlegel and Gill 1984; Schlegel et al. 1986). Therefore, as a complementary technique to C-banding, N-banding would be very helpful for the rapid identification of chromosome 2R, 3R, and 6R and/or 2RL, 3RS, and 6RL (Schlegel et al. 1986). With the intention of setting up a consistent system of the chromosome and band designation of rye, the Imperial rye is considered to have the standard karyotype. A standard nomenclature system was proposed during 'the 1st International Workshop on Rye Chromosome Nomenclature and Homoeology Relationships' (Sybenga 1983) and it was reaffirmed during 'the 2nd Workshop' in 1985. Thereafter, Schlegel et al. (1986) suggested another nomenclature system for C-band designations in rye. Subsequently Gill et al. (1991) proposed a nomenclature system for a description of

chromosome bands and structural aberrations in wheat, which has been followed in subsequent studies, including the detailed description of the C-banded karyotype of rye by Mukai et al. (1992).

Besides karyotyping, rye aneuploids have an important aspect in genetic and genomic analyses, mainly for mapping genes to chromosomes and chromosome arms. Almost the entire series of primary trisomics and telotrisomics of rye were developed successively by Kamanoi and Jenkins (1962), Balkandschiewa and Mettin (1974), Zeller et al. (1977), Pilch (1978) and Sturm (1978). However, it is not easy to use these trisomics and telotrisomics for genetic studies because they do not breed true and are highly sterile (Gill and Friebe 2009). Instead, a complete series of rye-wheat addition lines (Driscoll and Sears 1971) and telosomic additions of rye to wheat (Mukai et al. 1992; Li et al. 2013) have been important materials for rye genome mapping. In addition, recombinant lines between rye and wheat are also valuable materials for chromosome bin mapping (Rogowsky et al. 1993; Lukaszewski 2000; Lukaszewski et al. 2004). More importantly, so called rye dissection lines of common wheat, which carry various deletions and translocations of rye chromosomes induced by the gametocidal system, were developed in the previous studies (Friebe et al. 2000; Masoudi-Nejad et al. 2002; Tsuchida et al. 2008; Gyawali et al. 2009, 2010) and in the present work. These lines are especially precious resources for cytological or physical mapping of rye chromosomes.

In terms of molecular research aspect, i.e. the progress in genome analysis and the development of molecular markers, rye lags behind most of the other cereals. According to a report by Bartoš et al. (2008), there was no ongoing sequencing project in rye, and neither is there any such project at present to my knowledge, which was simply due to the underrepresentation of sequence databases. In rye, only 10,369 ESTs, in contrast to 1,575,304 in wheat and 556,070 in barley, are available to date. The lack of sequence information is a main limitation for marker development and gene cloning in rye (Bartoš et al. 2008). Although a certain number of markers were developed and used for construction of genetic map in rye (see Milczarski et al. (2011) and Li et al. (2013) for details), most of them were hybridization-based RFLP and

very limited number of them were PCR-based markers. Compared to RFLP markers, PCR-generated markers can be checked with a small DNA sample, without the use of radioactivity and without the time-consuming blotting procedure. Therefore they have broadly been used in genetic and genomic analyses. To date, a series of PCR-based markers have been developed in rye for genetic analysis and breeding programs (Saal and Wricke 1999; Brunell et al. 1999; Nagy and Lelley 2003; Isik et al. 2007; Kofler et al. 2008; Lee et al. 2009; Wang et al. 2009). However, the specificity of those markers to single chromosomes is not high. Above all, although 20 SSR markers (Saal and Wricke 1999) and 31 EST markers (Xu et al. 2012) were developed that are specific to individual rye chromosomes, the total number of markers is still far from enough to the genomic studies of rye. Recently, a mass of single nucleotide polymorphisms (SNPs) were surveyed (Li et al. 2011; Haseneyer et al. 2011), but they have not yet been genetically mapped to specific rye chromosomes.

In view of the above situation, it is remarkable that 110 rye specific PLUG markers were developed in the present study, and 79 were assigned to individual rye chromosome arms with a high success rate of 55%. Furthermore, through the analysis of comparative mapping between wheat and rye chromosomes in this study, the chromosomal locations of seven PLUG markers (TNAC1383, TNAC1280, TNAC1768, TNAC1291, TNAC1485, TNAC1711 and TNAC1929) revealed syntenic relationships between the rye and wheat that are different from those previously reported (Devos et al. 1993a; Martis et al. 2012). These new findings therefore suggested revision of the previous syntenic relationships. Based on the novel results and previous reports, I presume that an interchromosomal translocation occurred between the ancestral 3R and 6R chromosomes, followed by an inversion within the translocated 3R chromosome; the discrepancy of arm localization (chromosomes 5R, 6R, and 7R) might be explained by cryptic pericentromeric inversions. Besides, stable clear gaps were observed in most of the rye chromosomes stained with DAPI might be the imprint left behind by the chromosomal rearrangements occurred during the karyotype evolution. To be noted especially is that the number of such gaps is more than the number of the breakpoints of

chromosomal rearrangements inferred from the present and previous studies (Fig. 1-5). Therefore, the rye genome might have suffered from more complicated chromosomal rearrangements.

High-quality dense chromosome or genome maps are a very important tool for evolutionary genomics and numerous practical applications of genomic data like breeding and gene cloning (Korol et al. 2009). Chromosome-specific DNA markers in quantity are the primary factor in constructing high-density chromosome maps. Reviewed as above, a growing number of PCR-based markers have been developed in rye, but the number of the markers still cannot meet the demand for the construction of high-density maps in rye.

Flow cytometry or flow cytogenetics (FC) is a superior method capable of isolating single chromosomes and their arms with sufficient purity (Doležel et al. 2009, 2012). It provides a greater variety of uses, and the DNA of sorted chromosome is suitable for further molecular studies (Doležel et al. 2004, 2007). Using the FC method, all seven rye chromosomes were sorted with euploid rye and wheat-rye addition lines (Kubaláková et al. 2003). Then, Stein et al. (unpublished) sequenced all those sorted chromosomes individually. Based on the sequence information from the sorted chromosomes, more than 26,000 SSR primer pairs of rye were designed in this study. I randomly chose 2,112 SSR ones including 2,063 from non-genic regions and 49 from genic regions and, through PCR analysis using a complete series of wheat-rye addition and substitution lines, I successfully mapped 857 rye-specific SSR markers to individual rye chromosomes, about 120 markers per chromosome. Such SSR markers are surely invaluable in the development of chromosome maps, in the study of rye inter- and intra-species diversity, and especially in detecting rye chromatin or segments in the wheat background.

I. Future research directions

Using the critical lines (chromosome-mutation-inducing lines) and double disomic lines (disomic for both rye and Gc chromosomes) established in the present

study, we will be able to produce and establish as many rye dissection lines as we want. This will undoubtedly be essential to the construction of rye cytological maps, which will be useful to elucidate the structure rearrangements of the rye chromosomes.

Considering the high success rate of developing the existing 95 rye PLUG markers (16 different markers from Tsuchida et al. 2008) from the wheat PLUG markers, it will be a meaningful project to develop rye-specific PLUG markers on a large scale from existing wheat PLUG primers (Ishikawa et al. 2009; Saito et al. unpublished) and directly from rye ESTs and even barley EST information. Furthermore, it will be valuable to construct more detailed comparative maps between rye, barley and wheat using a sufficient number of PLUG markers common to the three crops, which would contribute to uncover more detailed and more real syntenic relationships among these crops. It would be an interesting and important subject to construct high-density genetic and physical maps using a mass of rye-specific SSR markers. It is also important to survey the transferability of SSR marker between rye, barley and wheat for the analytical study of the evolutionary relationships among the three crops.

Among the rye-specific PLUG and SSR markers there were some markers that were not allocated to any of the rye chromosomes by using the wheat-rye addition and substitution lines. This implies genetic polymorphism within Imperial rye. It should become an interesting and challengeable research field to focus on genetic polymorphism within a rye cultivar as well as between rye cultivars.

II. Summary

- Three new rye telosomes (2RS, 3RS, and 5RS) were developed in this study. Now, 13 out of 14 rye telosomes, except 3RL, are available.
- It was demonstrated that PLUG is a superb system of mining species-specific or chromosome-specific markers.
- The gametocidal system was proved to be able to induce chromosome mutations

or breakages to all rye chromosomes including 1R.

- Non-genic SSRs were assigned to individual rye chromosomes with higher frequency than genic SSRs in the genomic background of wheat.
- Out of 2,112 rye SSR primer pairs, 857 were mapped to single individual chromosomes.
- Of the 857 newly developed rye-chromosome specific SSR markers 187 (21.8%) had perfect SSRs with lengths of ≥20 nucleotides.
- The presence of genetic polymorphism within a cultivar, as well as between cultivars, should be considered in the genetic and genomic studies of rye.

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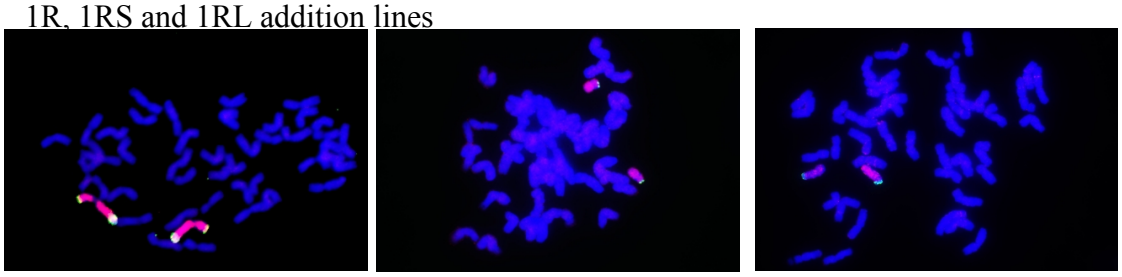
APPENDIX 1

Supplementary Figures

1R, 1RS and 1RL addition lines



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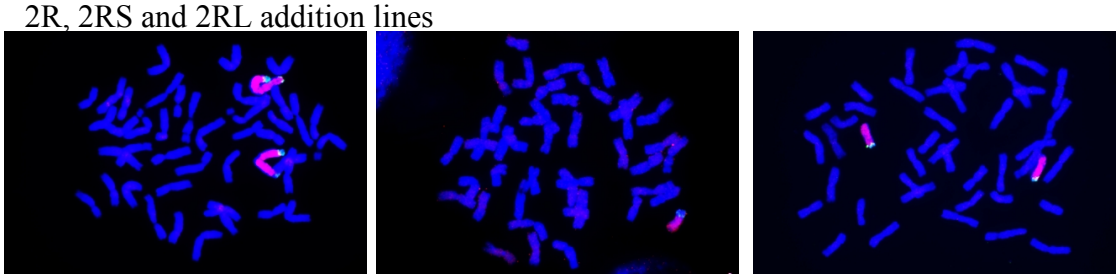

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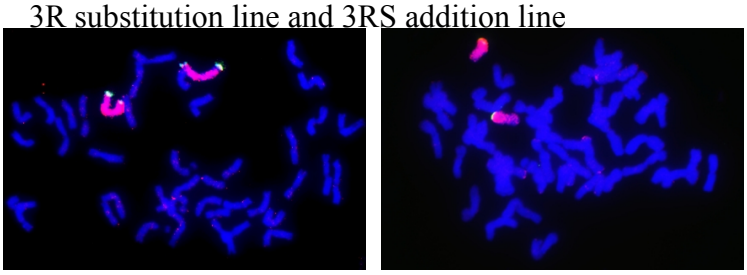

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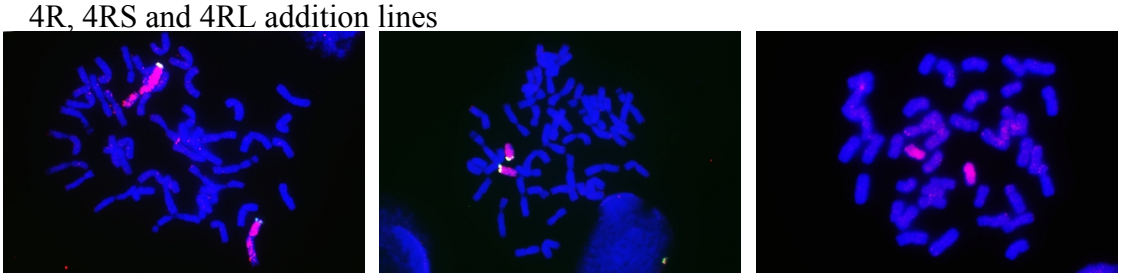


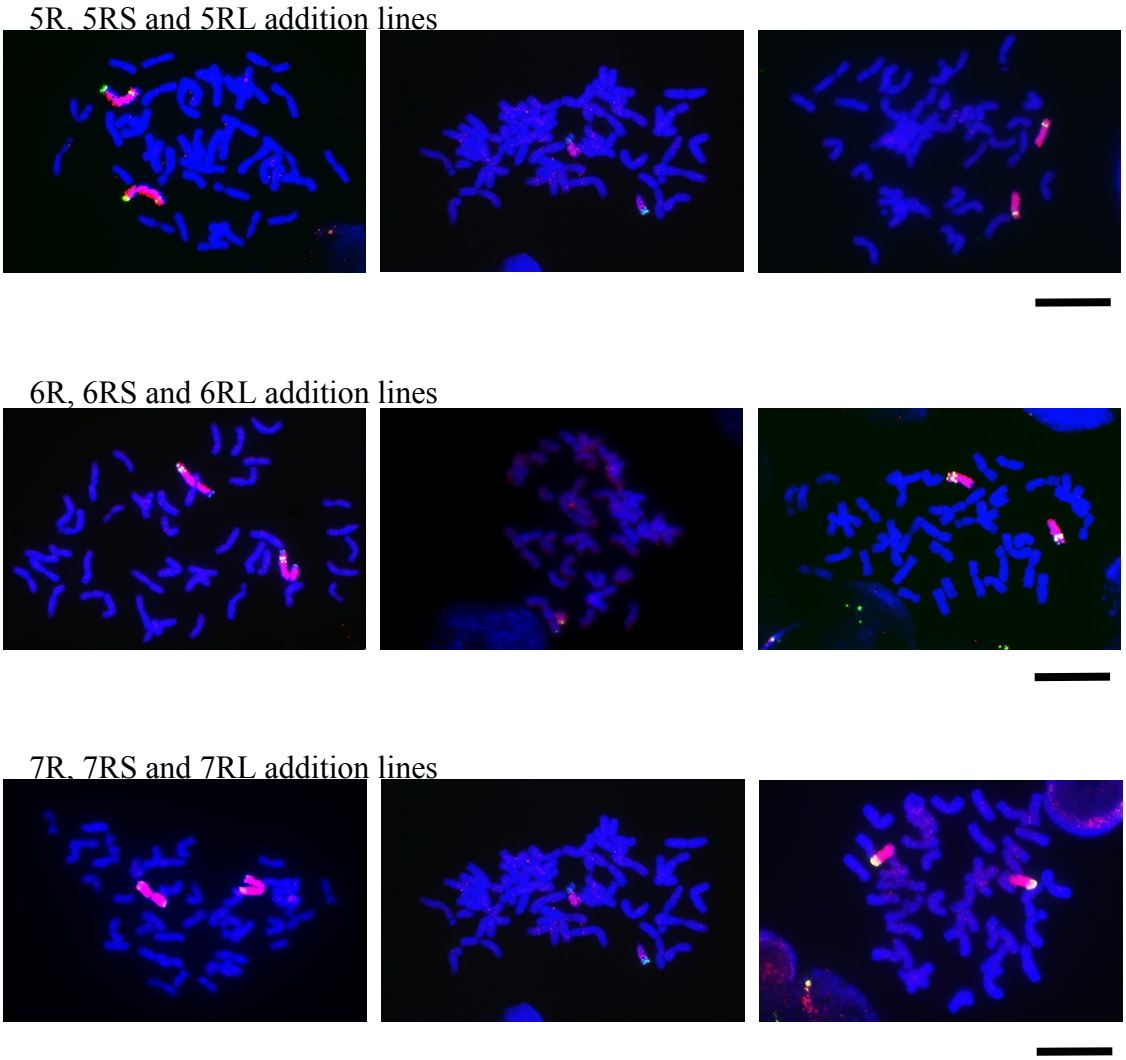
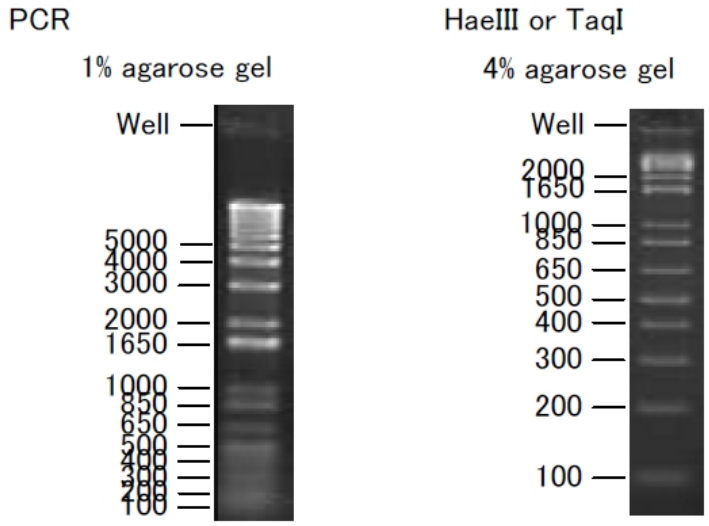
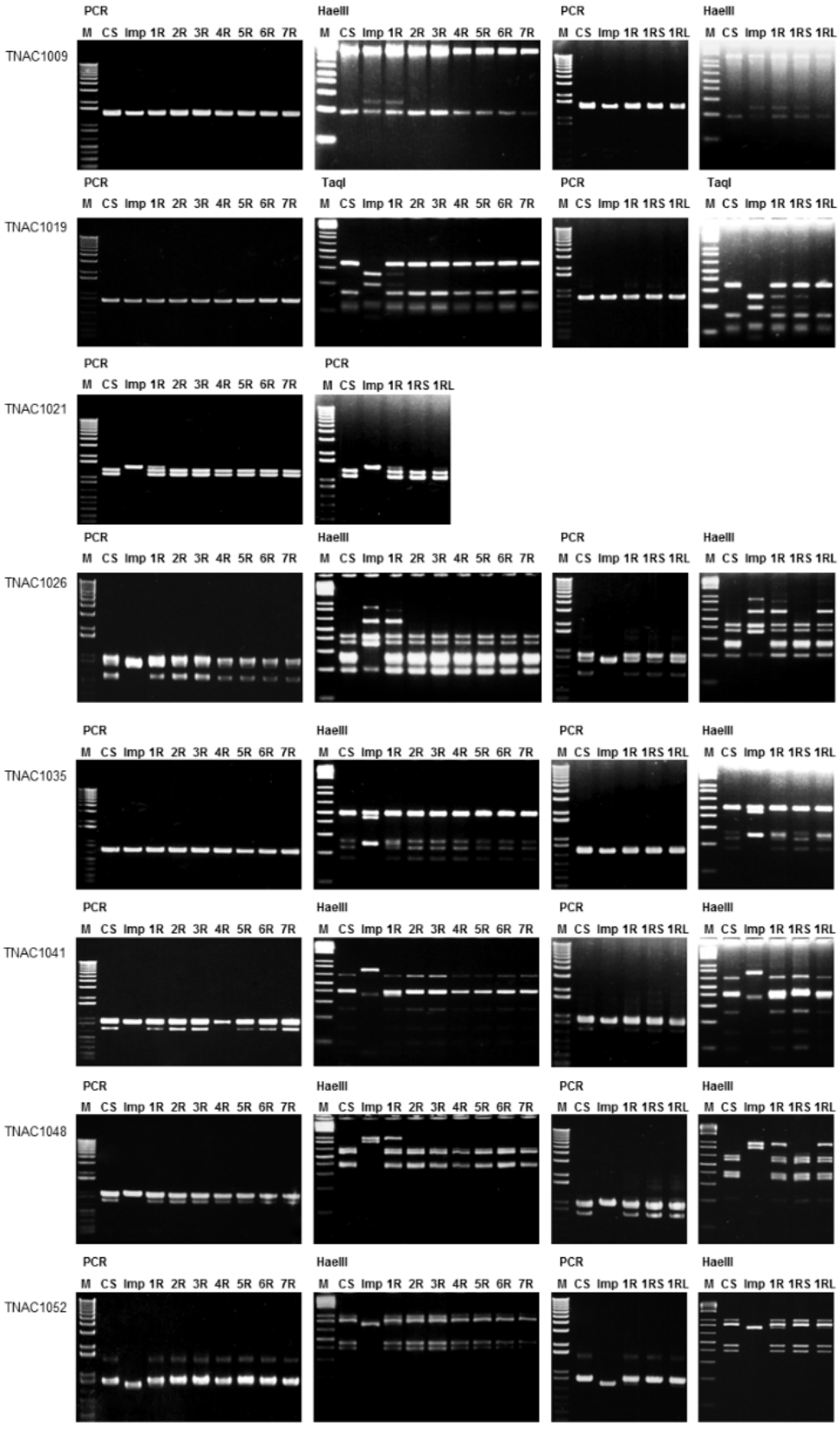


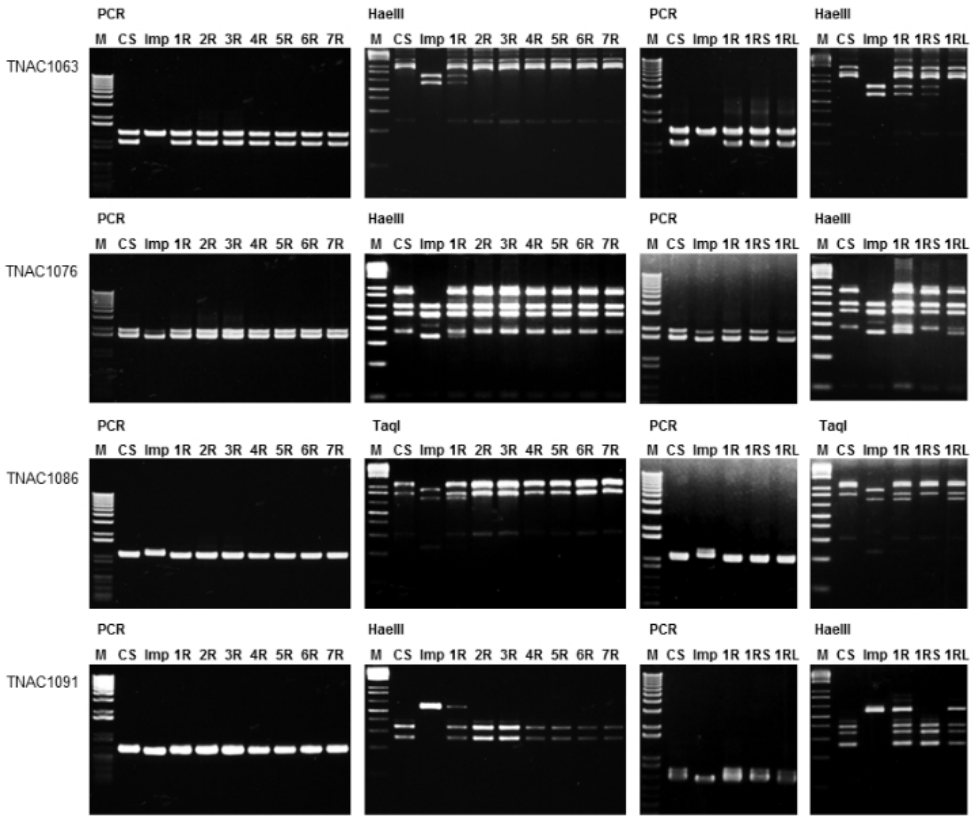
Fig. S1-1. FISH (*green*)/GISH (*pink*) photomicrographs of the mitotic metaphase cells of the wheat-rye addition and substitution (for 3R) lines. FISH probe: pSc200, GISH probe: rye total genomic DNA. *Bar*=10 μm.

Fig. S1-2 Gel-documentation for PCR analysis of the 84 rye specific PLUG markers assigned to its chromosomes and chromosome arms. Note that PCR products and restriction enzyme-digested PCR products were separated on 1% and 4% agarose gels, respectively.

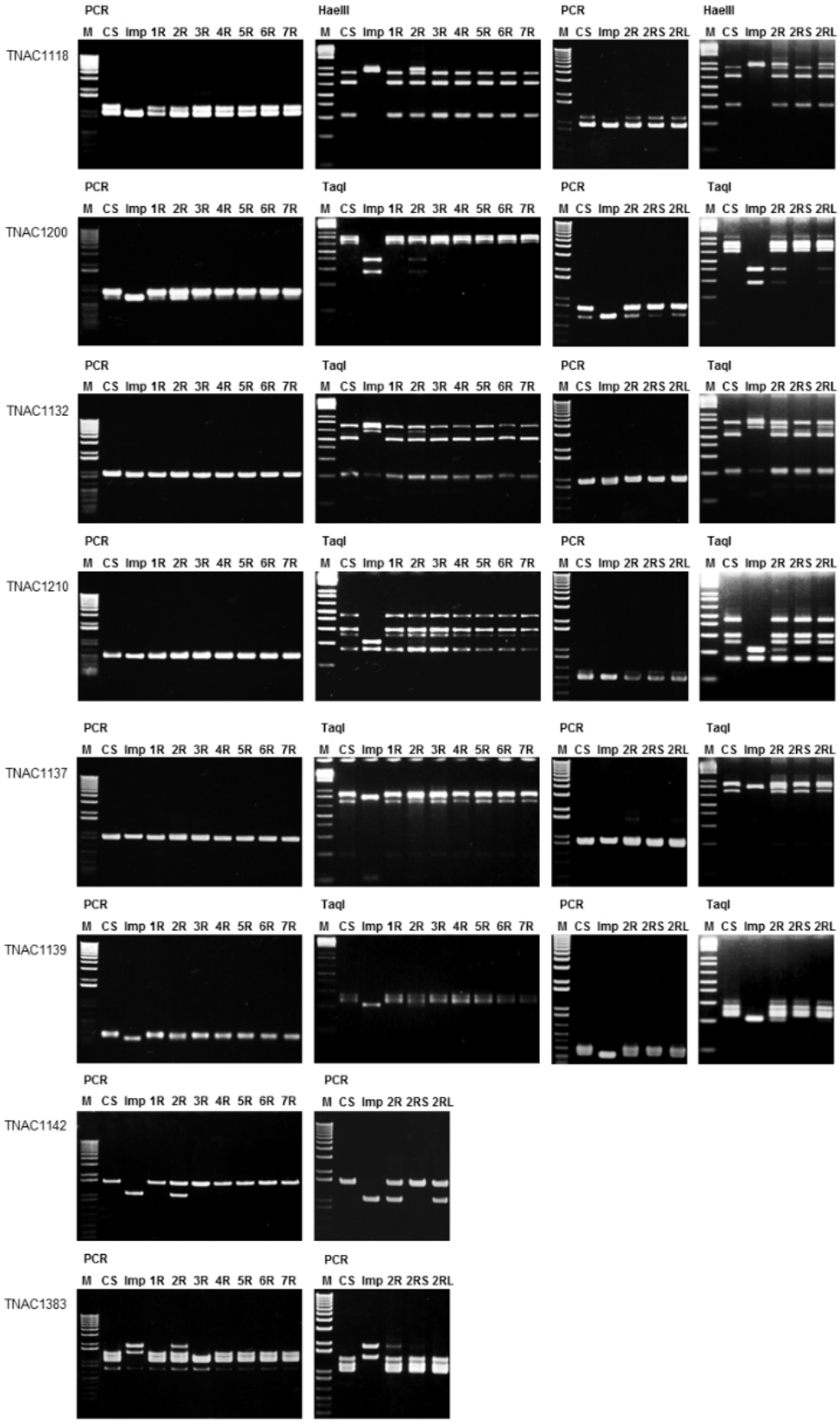


Chromosome 1R

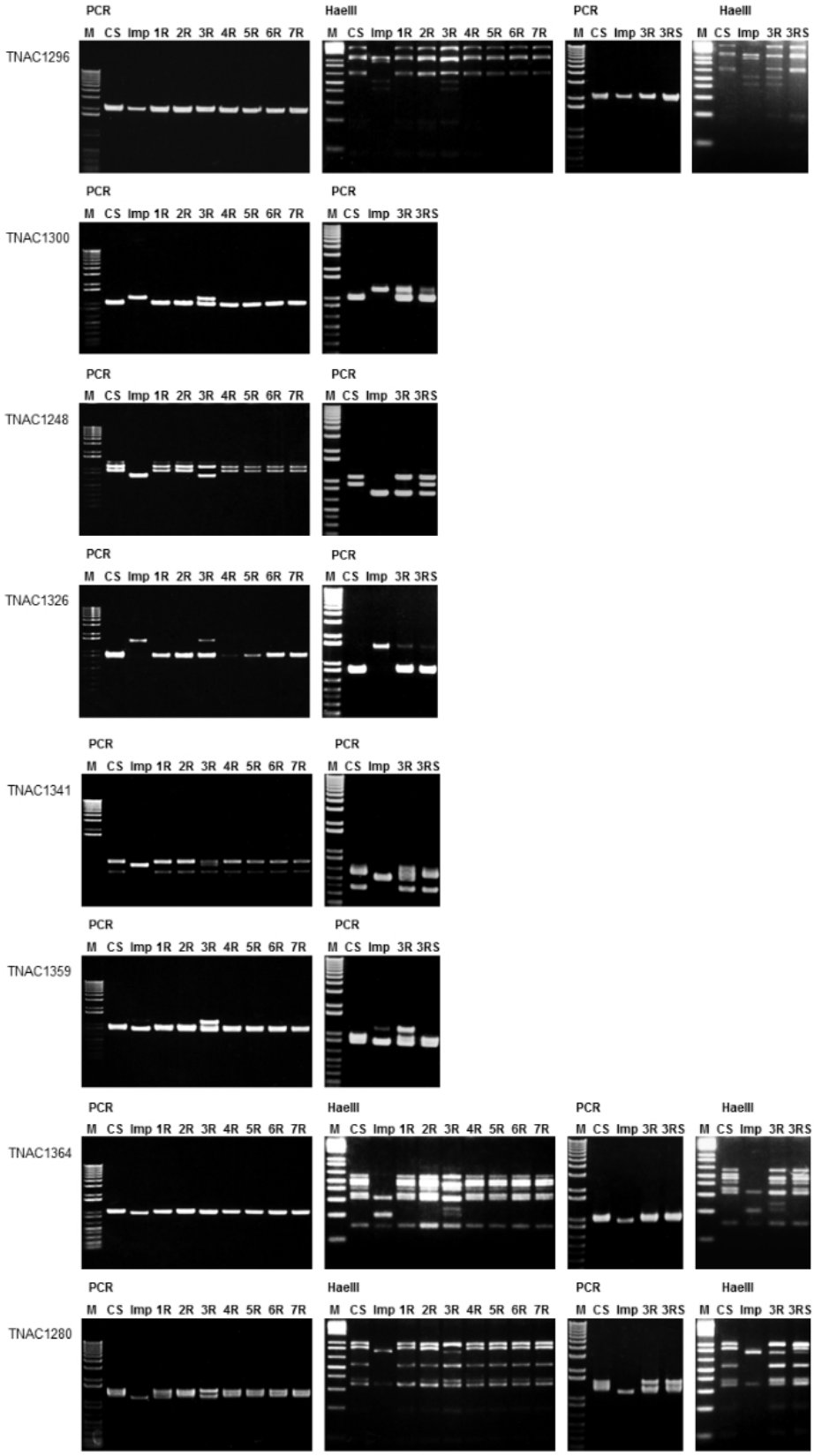


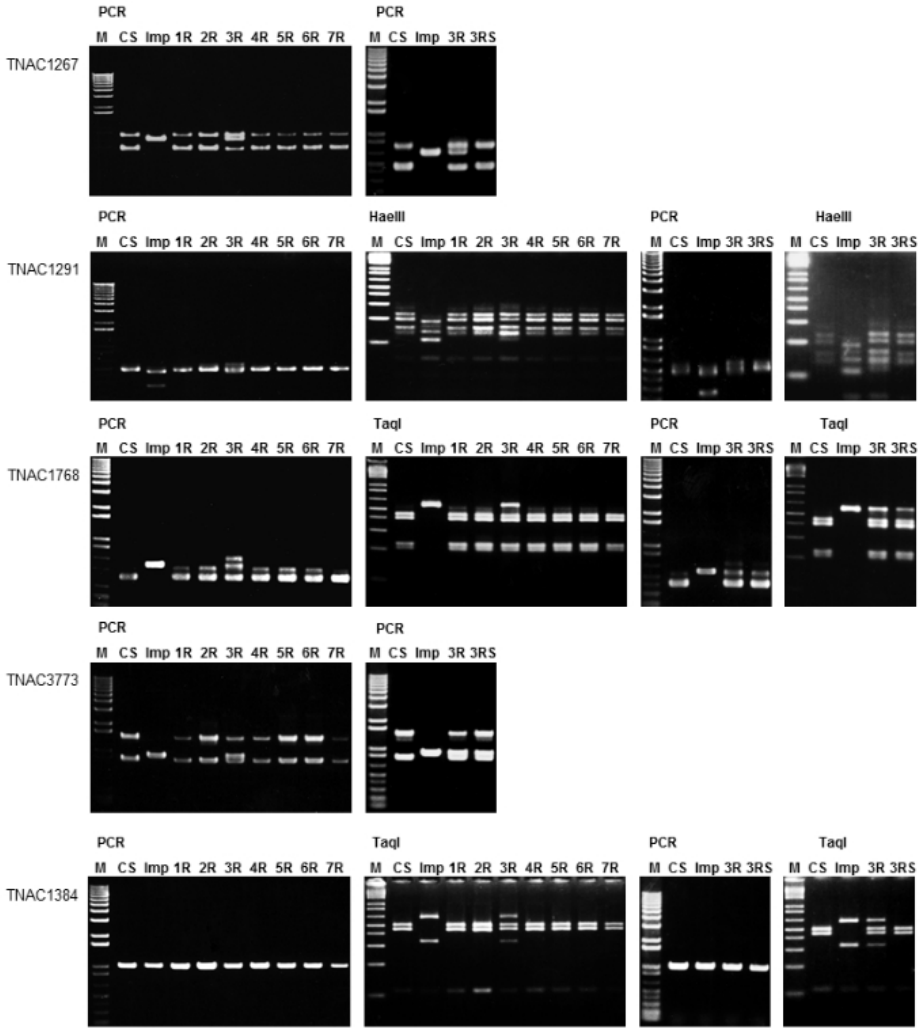

PCR
HaeIII
PCR
HaeIII
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 1R 1RS 1RL
TNAC1063
TNAC1076
TaqI
TNAC1086
TNAC1091

Chromosome 2R



Chromosome 3R

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PCR
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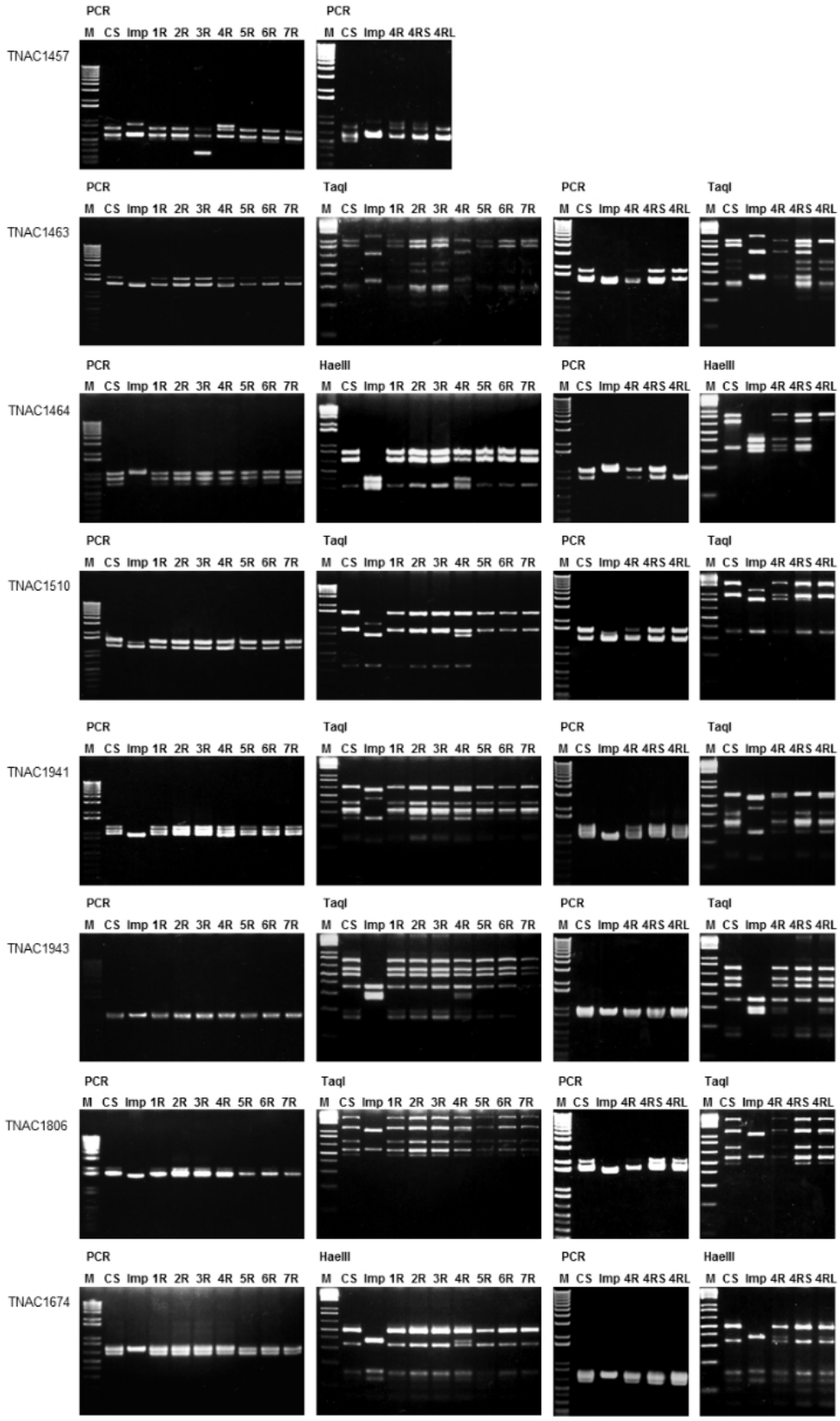
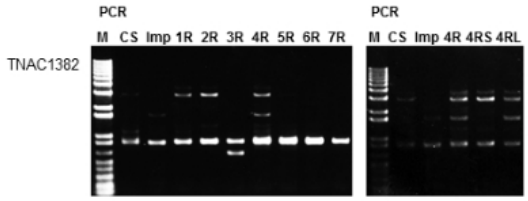
PCR
M CS Imp 3R 3RS
TNAC1291
PCR
M CS Imp 1R 2R 3R 4R 5R 6R 7R
HaeIII
M CS Imp 1R 2R 3R 4R 5R 6R 7R
PCR
M CS Imp 3R 3RS
HaeIII
M CS Imp 3R 3RS
TNAC1768
PCR
M CS Imp 1R 2R 3R 4R 5R 6R 7R
TaqI
M CS Imp 1R 2R 3R 4R 5R 6R 7R
PCR
M CS Imp 3R 3RS
TaqI
M CS Imp 3R 3RS
TNAC3773
PCR
M CS Imp 1R 2R 3R 4R 5R 6R 7R
PCR
M CS Imp 3R 3RS
TNAC1384
PCR
M CS Imp 1R 2R 3R 4R 5R 6R 7R
TaqI
M CS Imp 1R 2R 3R 4R 5R 6R 7R
PCR
M CS Imp 3R 3RS
TaqI
M CS Imp 3R 3RS

Chromosome 4R

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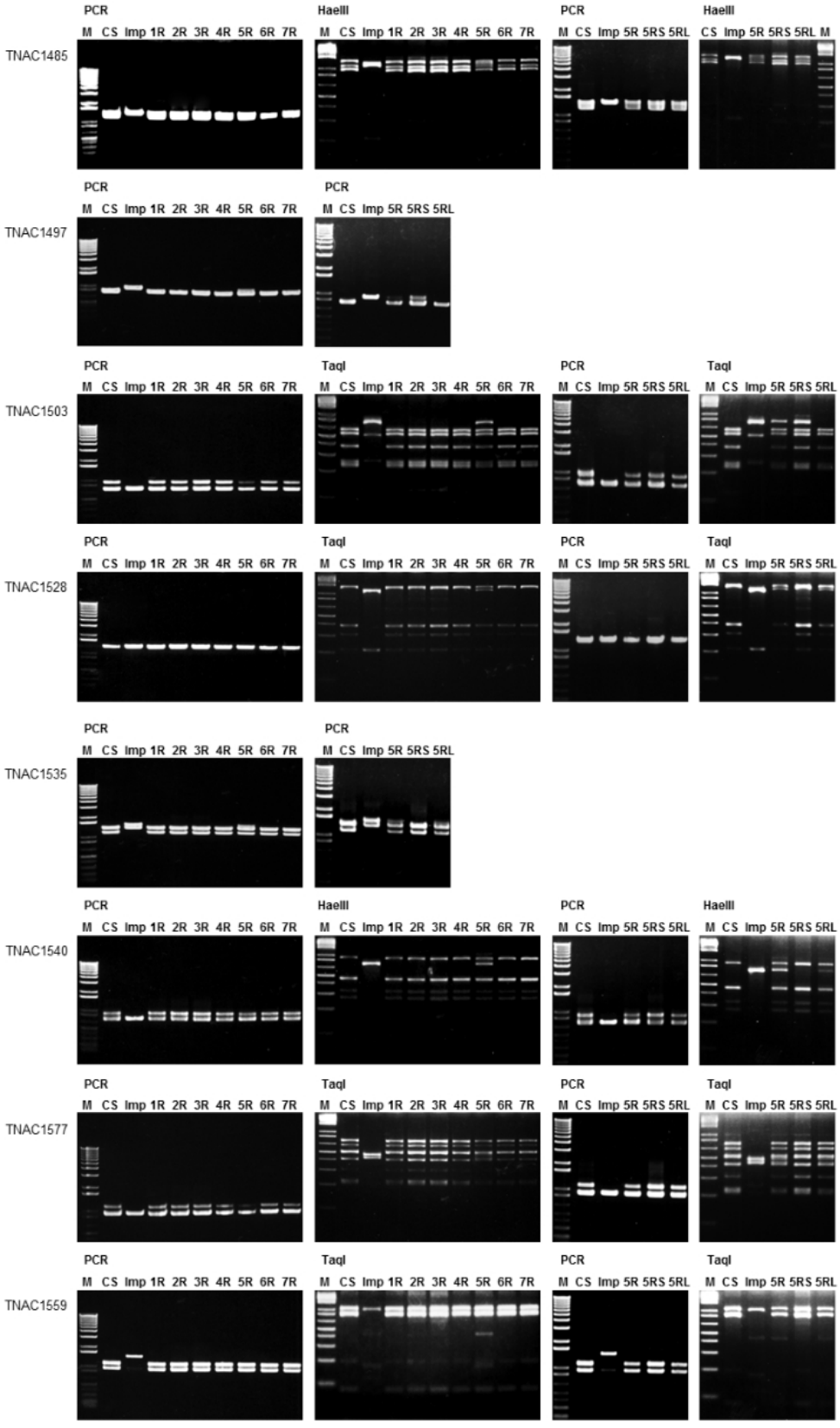
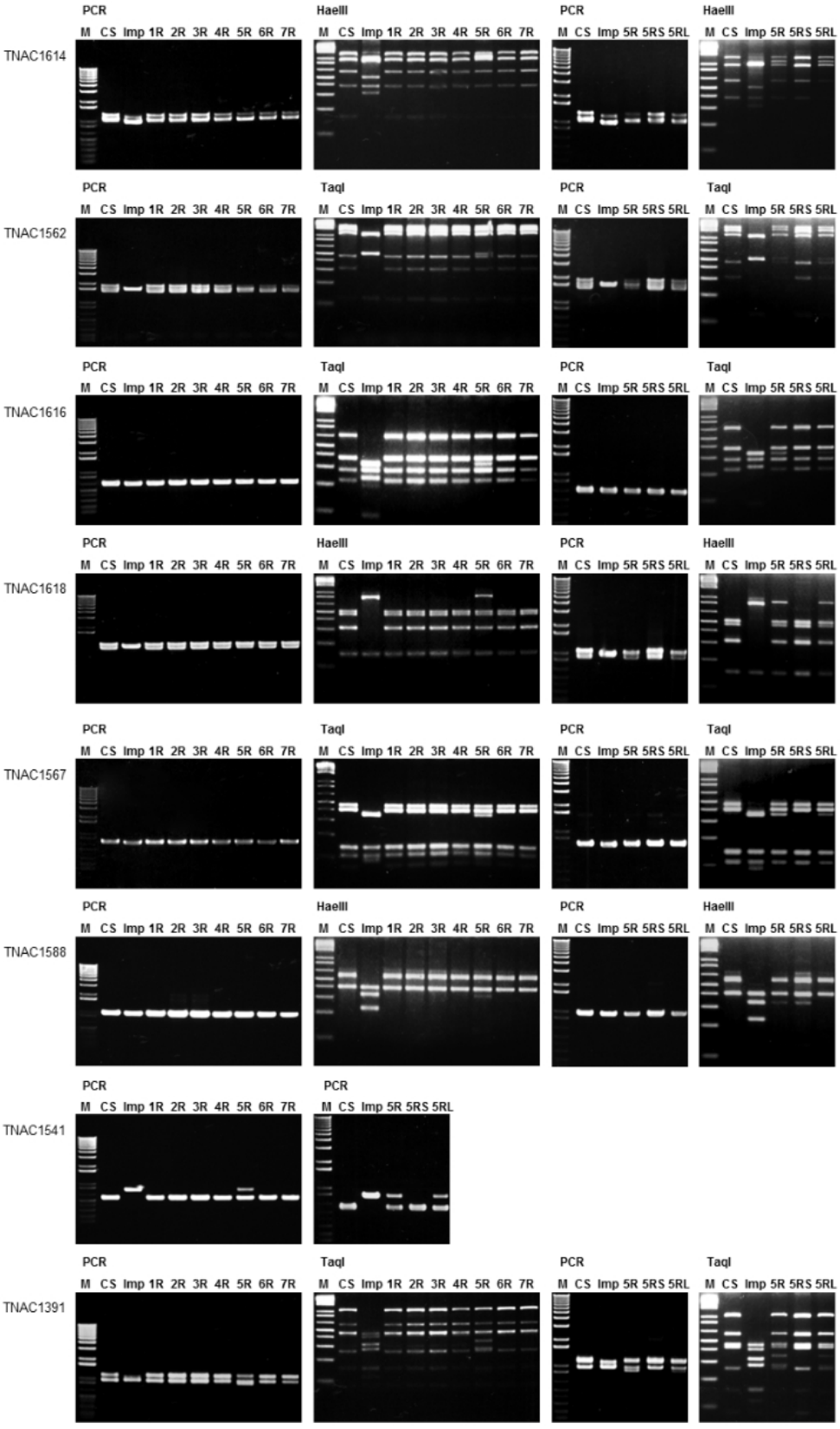
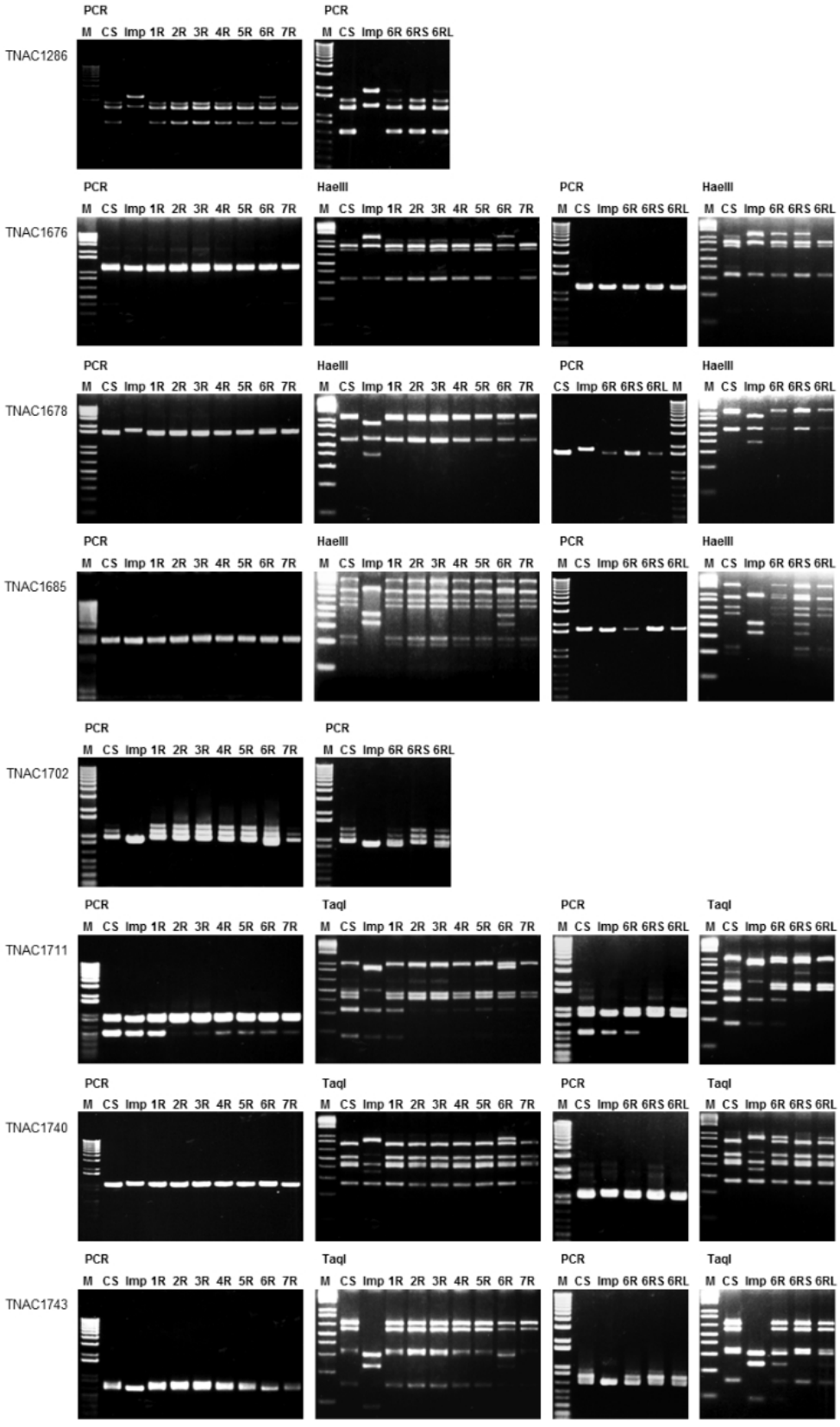
M CS Imp 1R 2R 3R 4R 5R 6R 7R
TNAC1382
PCR
M CS Imp 4R 4RS 4RL

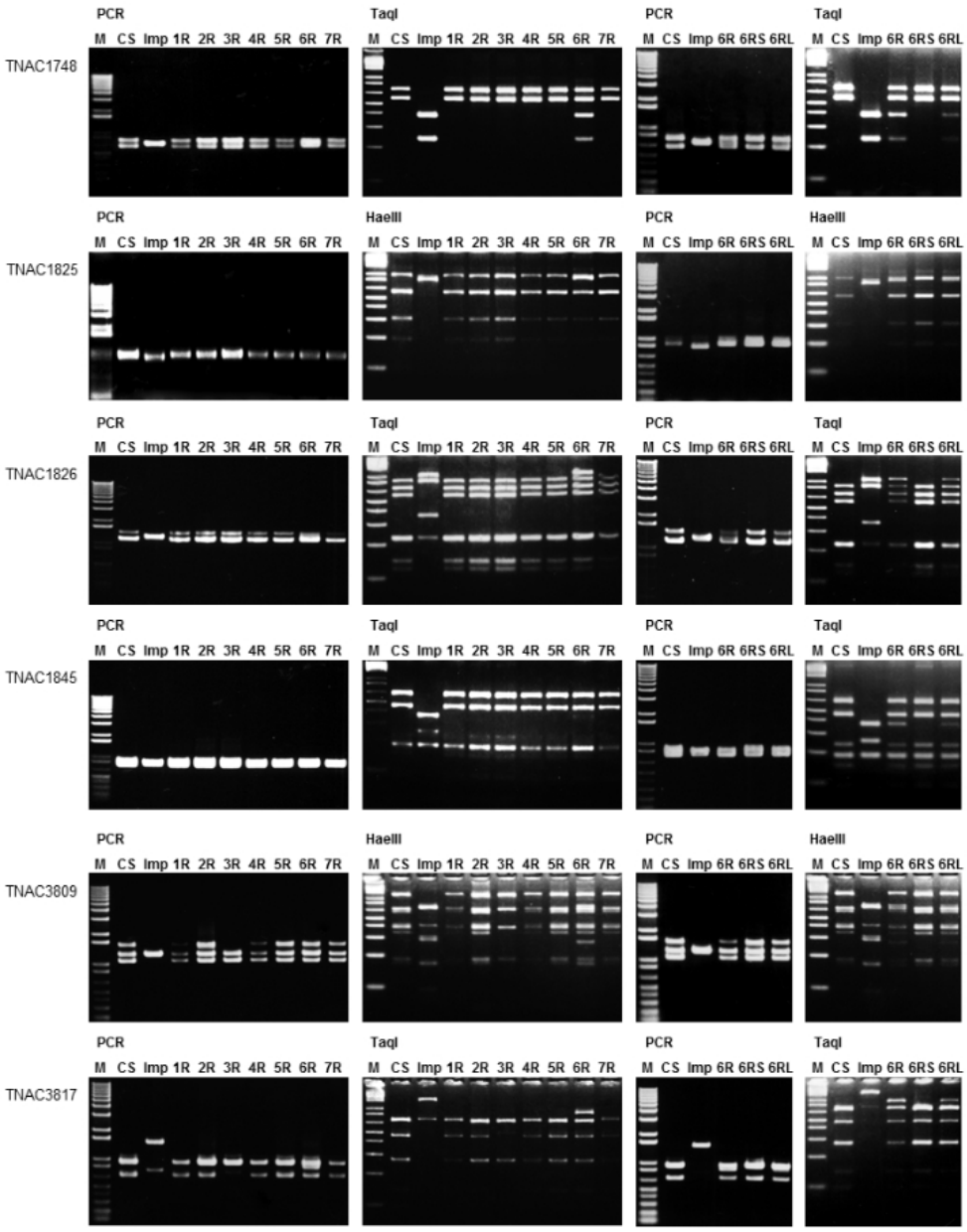
Chromosome 5R




PCR
HaeIII
PCR
HaeIII
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 5R 5RS 5RL
TNAC1614
PCR
TaqI
PCR
TaqI
TNAC1562
TNAC1616
TNAC1618
TNAC1567
TNAC1588
TNAC1541
TNAC1391

Chromosome 6R

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TaqI
PCR
TaqI
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M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 6R 6RS 6RL
M CS Imp 6R 6RS 6RL
TNAC1748
PCR
HaeIII
PCR
HaeIII
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 6R 6RS 6RL
M CS Imp 6R 6RS 6RL
TNAC1825
PCR
TaqI
PCR
TaqI
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 6R 6RS 6RL
M CS Imp 6R 6RS 6RL
TNAC1826
PCR
TaqI
PCR
TaqI
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 6R 6RS 6RL
M CS Imp 6R 6RS 6RL
TNAC1845
PCR
HaeIII
PCR
HaeIII
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 1R 2R 3R 4R 5R 6R 7R
M CS Imp 6R 6RS 6RL
M CS Imp 6R 6RS 6RL
TNAC3809
PCR
TaqI
PCR
TaqI
M CS Imp 1R 2R 3R 4R 5R 6R 7R
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M CS Imp 6R 6RS 6RL
M CS Imp 6R 6RS 6RL
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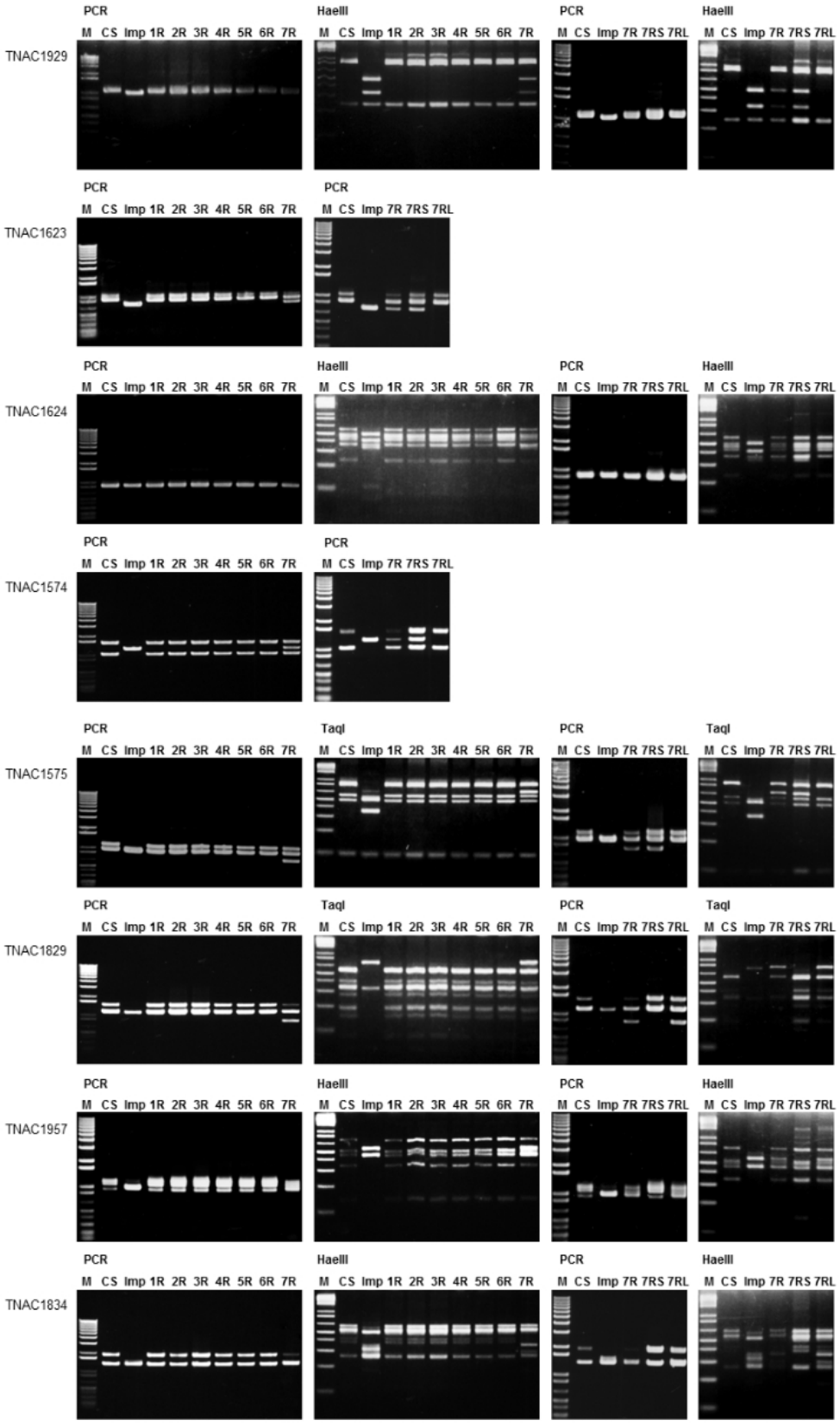
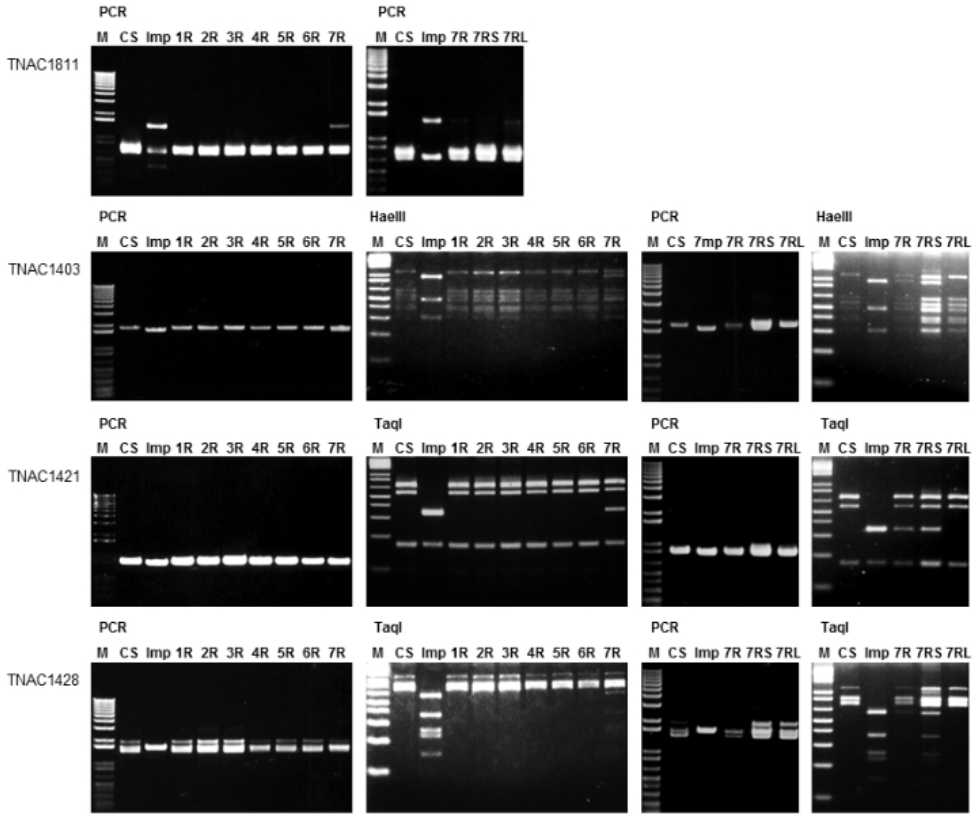
Chromosome 7R

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PCR
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PCR
M CS Imp 7R 7RS 7RL
TNAC1403
PCR
M CS Imp 1R 2R 3R 4R 5R 6R 7R
HaeIII
M CS Imp 1R 2R 3R 4R 5R 6R 7R
PCR
M CS 7mp 7R 7RS 7RL
HaeIII
M CS Imp 7R 7RS 7RL
TNAC1421
PCR
M CS Imp 1R 2R 3R 4R 5R 6R 7R
TaqI
M CS Imp 1R 2R 3R 4R 5R 6R 7R
PCR
M CS Imp 7R 7RS 7RL
TaqI
M CS Imp 7R 7RS 7RL
TNAC1428
PCR
M CS Imp 1R 2R 3R 4R 5R 6R 7R
TaqI
M CS Imp 1R 2R 3R 4R 5R 6R 7R
PCR
M CS Imp 7R 7RS 7RL
TaqI
M CS Imp 7R 7RS 7RL

APPENDIX 2

Supplementary Tables

Table S1-1. Wheat-rye addition and substitution lines used in this study

Rye chromosome/ chromosome arm	State	Source
1R	disomic addition	Dr. Lukaszewski
2R	disomic addition	Dr. Lukaszewski
3R	disomic substitution	Dr. Lukaszewski
4R	disomic addition	Dr. Lukaszewski
5R	disomic addition	Dr. Lukaszewski
6R	disomic addition	Dr. Lukaszewski
7R	disomic addition	Dr. Lukaszewski
1RS	disomic addition	Dr. Lukaszewski
1RL	disomic addition	Dr. Lukaszewski
2RS	monosomic addition	Developed in this study
2RL	disomic addition	Dr. Lukaszewski
3RS	disomic addition	Dr. Lukaszewski
3RL	not available	
4RS	disomic addition	Dr. Lukaszewski
4RL	disomic addition	Dr. Lukaszewski
5RS	disomic addition	Developed in this study
5RL	disomic addition	Dr. Lukaszewski
6RS	monosomic addition	Developed in this study
6RL	disomic addition	Dr. Lukaszewski
7RS	disomic addition	Dr. Lukaszewski
7RL	disomic addition	Dr. Lukaszewski

Table S1-2. Wheat PLUG primers to select rye-specific markers and their assigned rye chromosome-arms

PLUG[a] marker	wEST acc.[b]	PCR Primer		Rye-specific amplification[c]			Chromosome arm assigned
		Forward	Reverse	PCR	*Taq*I	*Hae*III	
TNAC1009	CV759922	CGAACGTGACCATCTACATCA	CATCTGACTTGGTCTTGGCATA			+	1RS
TNAC1019	CF134151	AACGTGTCCACCGTCTACATC	CCAGTGGTCTCTGATTCATCC		+	+	1RS
TNAC1063	DN949140	AGCCATTCACAGCTCTTCTTG	AATATGCTTCCTGGAGTCACG		+	+	1RS[d]
TNAC1021	BU100679	CTCATGCATGCGTTTGTTAAA	CCAGCTGAAACAAGCATCTTC	+			1RL
TNAC1026	CK168220	GGGATAGAACTCTGGGACTTCA	AGTGCCAGGGCATAATACAGC		+	+	1RL
TNAC1035	BM135164	TGCACTGGGATCTAACCTAAA	TCCAGTGATCATTTGAAGATTCC			+	1RL
TNAC1041	CK210333	TCACCACCTCTTTCAGTTGCT	GCATCAAGGATGAGGAGTCTG		+	+	1RL
TNAC1048	CK217172	ACTGAGGTAGAATCGCCACTG	GCCGCTATCGTCTGGTACAT		+	+	1RL[d]
TNAC1052	CK208771	TCAGCTGTGTCTGTTCTGTCCT	TGAGTCGACAAGACCACTCCT		+	+	1RL
TNAC1076	CK211671	GGGAGACGATCCTCTTATGATCT	TGCGTGCTTCCTAACTTACCA			+	1RL
TNAC1086	CK208179	CCAAACAAGATTGGATCATGG	CTGAGACCGTGCCCAGTG		+		1RL
TNAC1091	CV771105	CTCTTCTCCTTGGGACCTTTG	GAACAGGACCTGCTGTTTGG		+	+	1RL[d]
TNAC1383	CD939052	GCGGTCGATCTTCTTCAAGTC	TCAGATGGACTATGGGAGCAC	+			2RS
TNAC1118	DN949128	TTTCAATCAGCTCAACGGTTT	GGTGCTTGAGGGAGGTTAATG		+	+	2RL
TNAC1132	BJ258550	TATTGGTAGCCTTGTCGCTCT	TATGCTGCATGTGCTATCGAC		+		2RL
TNAC1137	CK171581	GCTGAATCACTCAACCATTCC	TGCTCGCGCTCTACTTCAC		+		2RL
TNAC1139	CD932542	ATGTTGTCCATGCCTCCACTT	CTGGAATTCTCCGTCTGCTTA		+	+	2RL
TNAC1142	BQ838874	GCCTACGAGTACATGGTCGAG	CAGCATCCATAACCAGGATGT	+			2RL
TNAC1200	BE405044	GTCCGCAGAATTGCTTCCTAT	TCTTCATGCATTGAGCGATAC		+	+	2RL
TNAC1210	CV770159	TTGTGACTGACAGCAACATCC	AGAGCTTGGCCTTCTCTTCC		+		2RL
TNAC1248	CK209182	ATGATGCAGCAGCAAATTACA	CTGAGGAGCCTCTCCAACTCT	+			3RS

TNAC1280	AB047554	CTCATTGGACCGAACAAAGTC	CAGAAGGTGCTCAAGCAGAG		+	+	3RS
TNAC1300	AY093953	TCTGCAGGTTCGGTAGACAAT	AGTACGGGAGGACGCATGT	+			3RS
TNAC1326	CK209388	ACAGATCGAGATGTTTATTGAAA	GATCAAAGAGATGCGCTGAAG	+			3RS
TNAC1768	CV765384	CCAGACGATACTGTGCATTCC	GGTGTCCAGCCAGAGTTTATG		+	+	3RS
TNAC1267	CK163058	GAGAGGCAGCTTCACTAGCAG	CGTCAGGATCAGCTCTCATGT	+			3RL
TNAC1291	CK161874	ATCAGCAAGCTCGTCTTCATC	TAGGCCCTTGTAGAGCGTGAT			+	3RL
TNAC1296	BJ301967	GCATCCTGTCCCTCATCAC	TCGAGGTCTCTAGACCAATGC			+	3RL
TNAC1341	CK209173	GTTGAAGCCTACATGCCACAC	TAGCATGGGCTCCTAACATTG	+			3RL
TNAC1359	BJ296725	GTAAATAGCGCCATCTGCGTA	CTCTGGATGCAGTTGGAATGT	+			3RL
TNAC1364	CK162104	CGTCAGGCTCAGGGTGTC	AAAGAGCCTCTGTCTCTCAGG			+	3RL
TNAC1457	CD929486	TTTGATTCCGTACTGCCTGAG	GCACCATTTGTTCCAGTCAAC	+			4RS
TNAC1463	CK166648	CGTCTTTATCAAACCCTGCAA	GTTCACCGAGTTCATCCAGAA		+	+	4RS
TNAC1464	CV771516	GGATGCCCTTACAAAGAGGTC	CGCAGACAGAAGTTAGCCAAG		+	+	4RS
TNAC1510	CK210745	GCGTCTGTCTTCATCTTCTGG	CAAGTGTGCACATGACTGCTT		+	+	4RS
TNAC1674	CD453605	CCACCACAGAAGCAGATGAAT	GCTAGATGGCACACCAAGTG			+	4RL
TNAC1806	BJ231361	ATTCCTCGTGAATTGCTGGAT	TCTGCAGTTAGGGACTTGAAA		+	+	4RL
TNAC1941	CV774188	AATGATCCTGACAAGGTGCAG	GTAGCGATGGCATCCAGAGA		+	+	4RL
TNAC1943	CK206453	GCTGCTATGGTCCACGAATTA	AGAGTATCGTATCCGGGCAAT		+		4RL
TNAC1497	CK217493	ATCAAACCTGACGGTGTTCAG	CATGCAGACTACAGGTCCAGA	+			5RS
TNAC1503	CK209224	TGAGGTTGGTTCTCATCTGGA	CGTTGGAAACAATCTGAATGG		+		5RS
TNAC1588	BQ578891	AAATCAGCAGGTGGCCAGTAT	AAATGGCGCACCATACTCAAG			+	5RS
TNAC1391	CK217716	GTACACGACCACCGAGGAAG	TGCATCTTTCGACCCTCATAA		+		5RL
TNAC1485	CK200624	CCCAAGTTCACTAACTTCGTTG	AAATAGTCCTGCATATCTCCTGT		+	+	5RL
TNAC1528	CK215417	TCGAGCTCATAATCAAATGGTG	AGAGGAAGGAACAGCCTCATC		+		5RL
TNAC1535	CK162425	TTCTAGTATCCGCGTTCTCCA	CAAAGCAATGCAGTCTTCCAT	+			5RL
TNAC1540	BQ838306	AACCTCAAGCACTGTCAGCAT	TTGCAGATCCTCTCAATCTCG			+	5RL

TNAC1541	BJ231692	ATCTTGTACCACCGTGAGAGC	GAGATCACGGTTTGCATCTGT	+			5RL
TNAC1559	AY847708	AAACAAGGCCCTGAAACACTT	CATTGTCAGGCTATGGGACAT		+	+	5RL
TNAC1577	BJ289348	CGCTCCATTTCCAGATCATAA	GCAGCATATAAATGGCCAAAC		+		5RL
TNAC1614	CK156789	AAACAGGGCTTTCAGCTTCTC	ATCAAGCAGAACAACCTCCAG		+	+	5RL
TNAC1562	CK194288	ACATCCGCAACAAGGAGTTC	GACTTCTTATGATGCACTATTCAGT		+		5RL
TNAC1616	BE430680	AGTCAAATTGCCATGTCAACAG	ACATCACCGCAGTAGGATTTG		+		5RL
TNAC1618	BT009403	GTTGGCTGTTGATGGTAAGGA	GGAGGCCACCAACTAATGTTT			+	5RL
TNAC1567	CA654435	ATGTTGGCTTTATACCAATGC	AGGTGCGGCTTCACTATCTTT		+		5RL
TNAC1676	BE413791	ATTGACGCGATTGATAGTGAA	AGTGGGCTACTGCTTCAGATG			+	6RS
TNAC1678	CV768357	AAATCTACTCTGCGAGGTTTGC	TGGGAAGCGCTTACTATTGTG			+	6RS
TNAC1685	CK197151	ATGGATAGCGGAAGCGACTC	AGCGTTTCCTCCGGTCTT		+	+	6RS
TNAC1711	BU672205	CTTAAATCGCCTTTCCCACTC	GACATTGCAAGGAGGAACAAG		+		6RS
TNAC1286	CV775292	TCCGGTGTTTGAAGAAACTTG	GTCCGGGAGGAGATCCAG	+			6RL
TNAC1702	CK157364	CATGGAAAGGTTGACAAGGAA	CTGGATGTTCCATTTCTGCTC	+			6RL
TNAC1740	CK206466	CGGAAGTGCTCGATTGTATCT	GCGGGTTTCTTCTCAACCTT		+		6RL
TNAC1743	BJ296943	CAGTTGATCAGGGCATTCAGT	TTCCAGTCCTTTGTTTCCACA		+		6RL
TNAC1748	BQ170604	TCGTAGAATTGGTCGACGATG	ATGGATTGGCAAAGAAAGATG		+	+	6RL
TNAC1825	CK211679	GAAGCGGTTCAGGGTGAC	ACCCATCACGTTGCTGTAGTC			+	6RL
TNAC1826	U34402	CACATATGATGATGACGGCAAT	GGCAGGGAGGAAACTCTACTG		+	+	6RL
TNAC1845	X94352	AATGAACAGCTTGCTTTCTGC	CAGATGCTCTGGATTTCATGG		+		6RL
TNAC1403	CV771781	CCTCCTCCATTGCGAGATAAC	GTAGTAACGCTGAAGGGTTCG			+	7RS
TNAC1421	CK165968	ATCCGCTTCTCCAAGTTCTTC	GTCCGATCCACTTCTTCAGGT		+		7RS
TNAC1428	CK171098	GCGTTGATCCTCAGAGAAGAG	TGAGAAGTCCCATGCAAATCT		+		7RS
TNAC1574	CD918266	CTCTTCTGCCTTGCTTGTGTC	GCAGAGTATCTGCCTGCTCAC	+			7RS
TNAC1575	BT009111	CAGCTCTAGCGAAAGACAAGG	AGCGATATTACGCCTTCCACT		+		7RS
TNAC1623	DR741726	TTTGGGTTGGCAAGAGAAGTA	TTCCAGGGAAGCATTAGGAAT	+			7RS

TNAC1624	CK209641	TTCTCCTCGAGAAGTTTGCAG	CCTCGTCACCAACTTCCTCTAC			+	7RS
TNAC1929	BT009094	GCACCAGAAGGTTCAGTAGCA	ATCTGTCAGCAGGGCACACT		+	+	7RS
TNAC1811	CK213680	CTGCTCAACGAGTTCATCGAC	TTGGAGTGGACGTTGCATT	+			7RL
TNAC1829	BQ838098	GCCACTTCCTCCCTCCTC	GTCGGTCCTCCAGTATCAGC		+		7RL
TNAC1834	BQ838710	CCGAGTGTCATCGTTAGGAAA	CCAGCAATCCTACCTGTTGAA			+	7RL
TNAC1957	CK162440	TCAACATTTGCAGGATTGTCA	TTTCACAGGAACCTCTGCATC		+	+	7RL
TNAC1001	CK162649	TTCCCATCTCTTGCCATTAAA	TTTCCGCTTCCTATGATGCTA				
TNAC1037	BJ228469	CCCTCAGTAATCGCGTAGAGT	AAGAGTTTGGATGCCATGAAC				
TNAC1038	CK202212	CCACCAGCTTTCCTTACCATA	ACTGCTCAATCCAACTGGAAA			+	
TNAC1043	BQ752788	CCCGAAGTTGATCTTTGTCTG	AGGGTCAAATTGAACGTGATG		+	+	
TNAC1044	CD874963	TCAGCAAAGTTCCAGAGAAGG	GAGGAACTCGTCGAGGAAGG				
TNAC1057	CK210128	GGAAGATGTGATGCCAACTGT	AAATATGCCGCCAAGTTAATG				
TNAC1071	BT009308	TGGACCTTCTGGTACGACATC	CTTCCCACTTTGGTTCTACCC				
TNAC1102	CK167138	GGAGAGGTGAAGGACCAACTC	CCTTGCAGCGTAGTGAGATTT		+		
TNAC1140	CV768000	TCCCAGAAATTACAAGGCTCA	AGGAACCCTATGCATTGGAAA		+	+	
TNAC1176	CK208960	CTTCATGGTTGCTCACGAACT	CATGCGAAATTTGCTATCCTT				
TNAC1178	TNAC1178	TGATACCGAGGCTATCCACAT	ACATGAACAAGGATCATGCTG			+	
TNAC1182	CK168408	CCTTCGTGCTAAGCTCACATT	CTCACCCACCTGTATGGATTG		+	+	
TNAC1183	DR740771	ACGCAGTTCATTTCTCTCCAA	GAGGCACACAGAATGACCTCT		+	+	
TNAC1195	CK163488	TGGTACAAGCAGATGGAGCAG	CAAGAGTCAGCGCAGAATACA				
TNAC1199	CK151983	AGTTGGGCCTTCAGCTAGGTA	TGGAAGTAAGAGAGAAGCAATATGA				
TNAC1204	CD913720	GAGAGGAATGCGTGAAGTTTG	AGACCATCTTTCCGGTCTTTG			+	
TNAC1233	CK167793	ATACCTCCTGAGCTTGGTCCT	CGACGTCGAATACTTCAAACTG				
TNAC1252	AY701776	AGCTCCGGCTTCTACCATTT	TGATGCACATGTTGCTATGCT		+		
TNAC1263	BE412000	TTGAGAAGTACCGCAAGGATG	CATCCACATCACTGTCACTGTC		+	+	
TNAC1277	CV768303	AAAGCACCACCACATATGAAA	GAGGCAGAGAGTGCAAATGTT				

TNAC1283	BQ743400	CGAGATACTGCACAGGAGAGG	TTCTTCCCTTATTTCAACAAGACC	+	
TNAC1301	AF543844	TGGTTTCAGATGCAGGAACTT	CACTAAGGCATGCTGAAGGAG		
TNAC1314	CK207209	AGGCTAAGGTGACGAGCAAA	TCATCATCAAAGCATTCACCA		
TNAC1356	BT009009	CGGCAAGTACTCCTTAACACG	GACGGTCGCGTACAACAAG	+	
TNAC1367	CV760737	CCTCAACATCTCCAAGGATCA	CCGCTGGATCTGATTAGGC	+	+
TNAC1373	CD880652	GTAGTGCCCATAACCGTCGTA	GACAGCGTCCAGAAGGAACT	+	
TNAC1398	CK170152	CAAGGCAGGTGCTGATATTGT	ACCCAGGGTTGACTGACATAA	+	
TNAC1408	DR739257	CAGGAAGTTGGTACCATTGTGA	CTTGCAGCCTCCTATTGATTC		
TNAC1412	CV760740	CTATGTCCGCAGCCATGAGTA	CTTCACACCATCCAAGCTTTC	+	+
TNAC1468	CK213665	GATGATCGCCAAGAAGTTGAG	CAGAACTCGCTGGAAATGATG		
TNAC1545	CK162209	TCCCTTCTGATTCAATAGCAGAT	CTGGGACCACTGCACACTACT		
TNAC1554	CK162719	TTGCTAGCTCAGCACAGTTTG	TTCTTGGTCACTCTGAGCGTA		
TNAC1605	CK207365	TTGCCCTTGTTGTGAAGAATC	TGTGCCATAGGCTCTCTTTGT		
TNAC1613	CK207248	CTCTTGTTTCCATGCCTTGAG	CTGGGTCATCCAGACTCACAA	+	
TNAC1627	CD374033	CAGGAGGCCTACGAGACG	TTCTTCAGCTCGGATATTTGG		
TNAC1644	BQ483694	GCCGCAGAGGTACTTGGTAAT	TGCTGCTGATAAAGCCACAAT		+
TNAC1656	CK153727	TTCCATGAGGAACTTGTCGAG	CCGGTCTCACGTCAGCTATT		
TNAC1663	CV768061	CAGATAGACCGGGTGGAATTT	CGAGGTCTACGTCTTCGAGTC		
TNAC1677	CK210485	CCCAAGAGATTTGGCATCATC	TGGCCATCACCTAAATGAAGA		
TNAC1679	CD865701	TATTGGCTCAACCAACCATTC	TTCCAAACCACCCAGTGTGTA	+	
TNAC1683	CD932392	CTTTCTCTTCTGCAGCTTGGA	CAGGCAGAACTGGTCAAGAAC	+	+
TNAC1726	CK206456	CTCAACATCCACGAGTACCAG	TTTGAAAGTTCCCAATCCAC		
TNAC1741	BE430390	GTCCCTGTTGGCTGTCTT	CTTGAGTGTCGCATCGGAAG	+	+
TNAC1751	DR738783	CTTCCTTTGCTTGTGATCCTG	GCCTGAGGACTTGAAGTGGTA		+
TNAC1752	CK158828	GTAGACGATGTCGAGGAGCAT	CTTCACCAATTTCTCCCATGA	+	+
TNAC1763	CK197357	CGATTGGCCGTACAACTTTC	TTGATGACGTTGAAGGGTCTC	+	

TNAC1776	AL815260	ATCATCCTGCTGCTACTGTGC	CCTTCTCAGCTTAGCGATGTG		+	+	
TNAC1781	CK168170	AACTGGCAATCAGCAGCAC	CACCACGCTCTCTTTCATCTT				
TNAC1782	CK205844	TCACTGAACAGCCTAGACATGG	ATTCGCAGACCGCATCTATC				
TNAC1787	CD491198	AAGTCCAGTGCTGGAGGATTT	CTTATGCCGCAAGGAGTTTCT				
TNAC1803	CK208410	TGCGACCAGTCTCTTTGAAAT	GTCGGAGCCTGGATCTCTAGT		+	+	
TNAC1805	CK162566	TTCTTTGCTGGTCGTTTCTTG	CCACGGTAATGTAAGGCACAT		+		
TNAC1812	CK167522	ACTTCGCTTGGTCTCCTCAAT	GAGAAGTGTGCCAATTCCAAA				
TNAC1815	CD873670	AGCAGACATCAGCAAGTTTGAG	ACTGACAAGCCCATGATTGAC		+		
TNAC1821	BJ249253	CACTATGGGCTCTTCAATCCA	CAGCCAAATAGTGTGCAATCC		+	+	
TNAC1822	BT008980	CCCTCCGTCCGTGCAAAT	GGCTGATGATGGAGACGTG				
TNAC1864	BQ281197	CAAATCTTCTGGCTTCTTCCA	TAGATAGTTCTTCGCGGCTTG				
TNAC1867	CK161622	GCCTTTCCTTTGGTAGTCTGG	CGATCCAAATGATCCTGAAGA				
TNAC1868	BT008921	CTCCGCCTTCATCGGAAA	CCGTTCTGCTTCAGGATCTC				
TNAC1888	BE418304	AGGGATGTGTTGGAGCTGTTA	CACAGTGACCTTCTGCTCCTT		+	+	
TNAC1903	BJ320311	TCGCTTCTTCTGCTTGTTCTT	CTGCTACTAGGCCACCCAAA				
TNAC1917	AF438328	TGATACTGTGCCAAGGTTTCC	ATGCAGGAGGCCTCTATCAAG				
TNAC1926	BT008966	CGTCAGCTACAGCGACATCTA	AACTTGAGCAGCGTGGTGTT				
TNAC1948	CD491203	TTTGTCTGTTAGGGCATCAGG	GTGTATGATGCGAATGGAAGG		+	+	
TNAC1956	BQ841856	ACGAAGGACAATTGCTGCTAA	GTGCACTTCTTGCCCTACTTG				
Confirmation of the rearrangement involving the long arm of G3							
TNAC3773[e]	AK331402[f]	CGAGGTTGTTCTCTCTGCTG	GTCTCCGGTGTTGACCAAGT	+			3RS
TNAC1384	CK207934	CTCCTGCTTCGCTGAGGT	CCATCTGCTTGCAATCTCACT		+		3RL
TNAC1382	CK151489	CCTGAAGGCTGTGAGATGCTA	ACCGATGGCACCACCAAG	+			4RL
TNAC3809[e]	Z12616_1	TGCGTCAGAAACAGGAAAGA	TCCTTGTAGTCCAGAAACTCCT			+	6RL
TNAC3817[e]	CJ552695	GCAAGGAAAACCAGCTGAGG	TTCGGTTTGCTTGTGCTTTT		+		6RL
TNAC3763[e]	CA600406	TGTGGACGGATTATGGCATT	TCAGGTTCGATCTTAGTTCATCAT				

TNAC3775[e]	AK333770[f]	ACTGGTGGGATCAAGGACAC	CCTGCTGGTTCGGTATCAAG	
TNAC3776[e]	CD882173	ATGGACGTGGAGACGATGA	CCTGGAGCTCATTCTCCATC	
TNAC3790[e]	CK204809	GCACCGACCATTGACAGACT	CGAGAATTCCAGCGATCTTC	
TNAC3793[e]	CJ543310	TGCTTCCGATTCTTGTGATCTA	CCCACAGGTGTCGTATGTG	
TNAC3794[e]	CK166632	GCCACCCATCAGCACAAG	GCAGCGAGAACCTGTAGCTC	
TNAC3798[e]	GH732116	TTATGTTGGCCATGTTACGG	CGCAAACCGACTGATGAAGT	
TNAC5091[e]	AK331402[f]	GAAACTGGACGCAGGGTATC	TGTGATCTTCCGAGATTCCAG	
TNAC7803[e]	AK331402[f]	CAGTAGGTGTTCCCAACACGA	TGCAAGTTCTTCAGCGTGTG	+
TNAC3818[e]	BM137927	CCTGAGGTACTCGGGATACG	ACCCGTCAGGTCGTTGTCTA	
TNAC7804[e]	BM137927	CATCTGCTCGTGAAGCTCCT	CCCGTCAGGTCGTTGTCTAT	
TNAC1288	CV779915	ACGAATGCTCTCGACCAAGTA	TTGAGGATGGGTCTATGGATG	

[a] Name of PLUG marker cited from Ishikawa et al. (2009).

[b] GenBank accession number of wheat EST.

[c] "+" indicates the occurrence of rye-specific PCR products in non-digest (PCR), or TaqI, HaeIII digestion.

[d] Markers TNAC1063, TNAC1048, and TNAC1091 were miss-assigned to chromosme arms by Tsuchida et al. (2008), and corrected in the present study.

[e] Markers were provided by Saito et al. (unpublished data).

[f] PLUG markers were designed according to the wheat full length cDNAs (Mochida et al. 2009).